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Factors Influencing Western Gall Rust Infection on Lodgepole Pine and the Seasonal Development of the Mycoparasite *Scytalidium uredinicola*

By

Bruce David Moltzan

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Factors Influencing Western Gall Rust Infection on Lodgepole Pine and the Seasonal Development of the Mycoparasite *Scytalidium uredinicola* submitted by Bruce David Moltzan in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Forest Pathology.

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ABSTRACT

Western gall rust caused by *Endocronartium harknessii* (J. P. Moore) Y. Hiratsuka is common on *Pinus contorta* Dougl. ex Loud. in western Canada. A study was conducted to determine factors influencing rust infection and seasonal occurrence of the mycoparasite *Scytalidium uredinicola* Kuhlman *et al.* Spore viability remained above 90% in both field seasons indicating that pathogen viability does not limit infection. Field shoots elongated to 90%, 95%, and 100% of their final length by the third and fourth week of June, and first week of July, respectively. Susceptibility at these stages was 100%, 60%, and less than 10%, respectively. The decline in spore production and decrease in susceptibility combined to reduce infection once shoots reached 90% of their final length.

The likelihood of *S. uredinicola* presence increased by a factor of 1.3 for each year of growth and increased by a factor of 2.1 for each 1 cm increase in gall size. The incidence of *S. uredinicola* was only weakly associated with rust severity in the stand. *Scytalidium uredinicola* was isolated from the surface of galls and from tissues beneath the periderm throughout the year on galls six years of age or older. Histological study confirmed the presence of *S. uredinicola* in unruptured sori as early as 24 April 1994. *Scytalidium uredinicola* reoccurred in the same location over 3 successive sporulating seasons from 1992 to 1994. On parasitized galls, *S. uredinicola* reduced rust spore germination to 5.5%.

Given that *S. uredinicola* is active at the beginning of the growing season when lodgepole pine is most likely to be infected by *E. harknessii*, and that it greatly reduces rust spore viability, its potential as a biological control agent would appear quite great. However, the weak association with rust severity in nature suggests that it may not be an important regulating factor in the pathosystem. Further, failure to establish on young galls may be a significant disadvantage for its use as a biological control agent.

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This dissertation is dedicated to my wife, Kristi, and my two daughters, Charvel and Amanda, who have enriched my life and endured the long pursuit of my higher education. I am truly blessed to continue to have Kristi's love, patience, and encouragement in the pursuit of my dreams. This work could not have been completed without her. I would also like to dedicate this thesis to our parents Floyd and LaVern Moltzan and Richard and Arlene Hammer. Their continued support over these past years has served as the foundation of which great people are made, and we will always remember the significance of their love. Finally, I wish to dedicate this work to the memory of my two friends, Ron Koech and Cliff Opheim who taught me a great deal about life.

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CHAPTER 1

A LITERATURE REVIEW OF *ENDOCRONARTIUM HARKNESSII* ON *PINUS* AND THE MYCOPARASITE *SCYTALIDIUM UREDINICOLA*

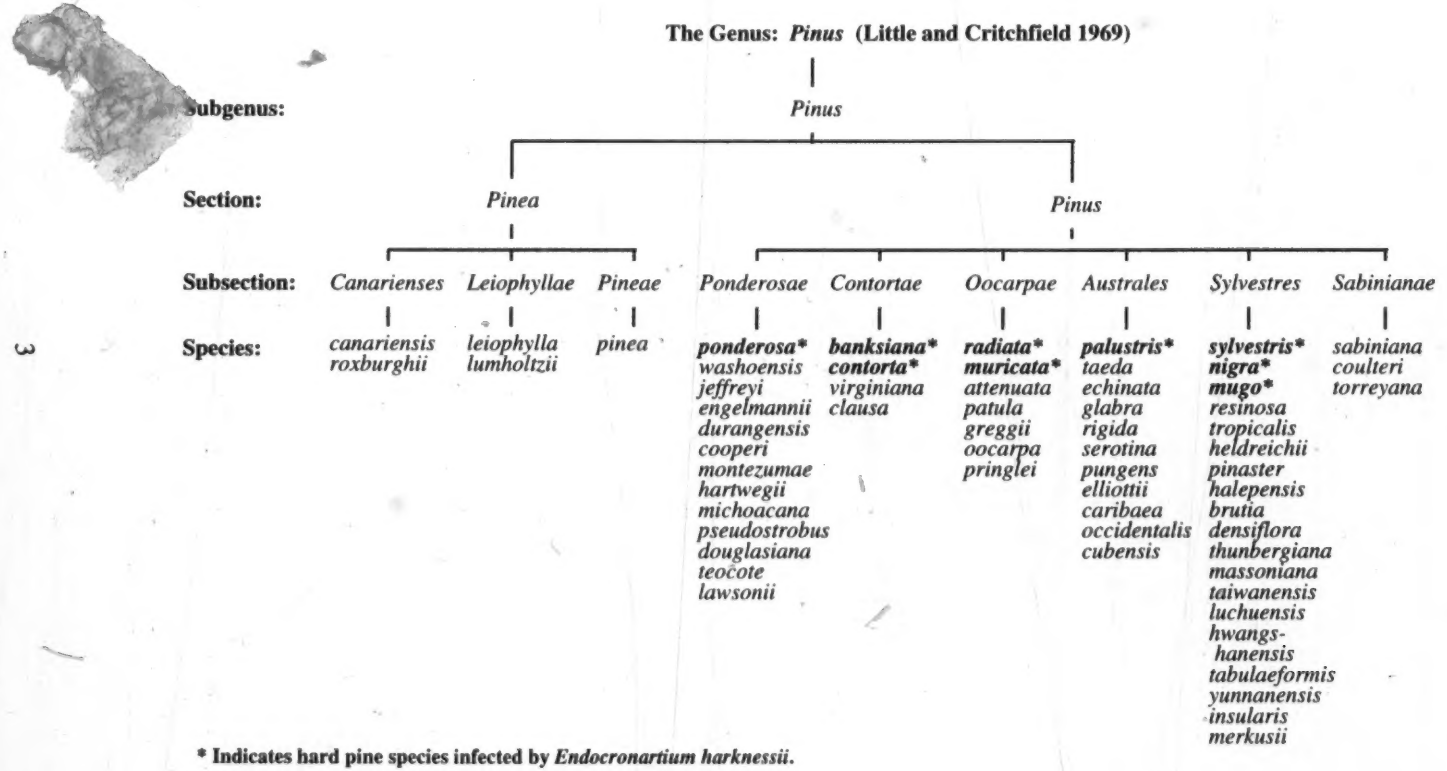
1.1 INTRODUCTION

The genus *Pinus* contains many species that are commercially important worldwide. Form, versatility, wood quality, and availability are key advantages for their use in the forest products industry. Ecologically, hard pines such as *Pinus ponderosa* Laws. (ponderosa pine), *P. banksiana* Lamb. (Jack pine), and *P. contorta* Dougl. (lodgepole pine) act as pioneer species that are vital to forest succession and reforestation. A disease that severely alters the form and limits the full growth potential of hard pines is western gall rust caused by *Endocronartium harknessii* (J. P. Moore) Y. Hiratsuka. This literature review includes a discussion of the suscepts, importance, biology, and control of western gall rust and a review of lodgepole pine development, periderm formation, and the mycoparasite *Scytalidium uredinicola* Kuhlman *et al.*, as an introduction to the overall investigation.

1.2 SUSCEPTS

The genus *Pinus* was first recognized by Virgil (70-19 B. C.) to refer to the pines in his works *Georgics* and *Ecloques* (Abbe 1965). The genus is found in northern temperate zones and tropical mountains world wide. Pines have been described as resinous, mostly evergreen with a straight axis and narrow crown. Wood is soft and lightweight (Little 1980; Little and Critchfield 1969). The bark may be furrowed into ridges or fissured into scaly plates. Pines are monoecious, having both male and female flowers on the same tree. Male cones are numerous, small, and clustered. Female cones are large, woody, solitary, and composed of many flat cone scales above each bract. Two long-winged seeds typically form at the base of the cone scale (Burns and Honkala 1990; Millar and Kinloch 1991). The modern authorities Critchfield and Little (1969) have divided the genus into 3 subgenera, 4 sections, 15 subsections, and 94 species. The subgenus *Pinus* alone comprises 2 sections, 9 subsections, and 62 species (Fig. 1.1). Two groups known as 'soft pines' and 'hard pines' have been delineated based on morphological and anatomical differences.

Figure 1.1. The subgenus *Pinus* divided into sections, subsections, and species based on Little and Critchfield (1969).



1.2.1 *Host development*

The annual growth of pine begins in spring when the apical dome of the shoot starts to divide and elongate, as dormancy breaks. Telescoped shoots, or long shoot buds contain cataphylls (scales), lateral branch buds, seed cones, dwarf shoots, and pollen cones, which formed during the spring and summer of the previous year (Lanner 1976; Owens and Molder 1975). Cells formed at bud set of the previous year begin to expand and form a vegetative shoot. In a 'wave-like' manner, tissue maturation begins first at the base of the long shoot, then progresses upward to the distal end. Needle primordia push out from between the cataphylls, and elongation of both needles and shoots is completed early in the summer (Owens and Molder 1984; Thompson 1976).

By midsummer, basal development of axillary buds increases in the same fashion as above, resulting in the formation of pollen cones or short shoot buds before the onset of dormancy. This is followed by the production of either seed cones or long shoot buds. The development of shoots and needles follows a sigmoidal pattern that can be increased or decreased by fluctuating temperatures (Owens and Molder 1984). All shoots develop scales, which surround the buds prior to dormancy. Sterile cataphylls that established at the end of the growing season protect the terminal bud over the winter. This process is typical for monocyclic buds produced on less vigorous branches in lower portions of the tree (Lanner 1976). Polycyclic buds are characterized by two or more sequences of development which occur in the same growing season. It is known that polycyclic buds are formed on vigorous branches in the upper portions of the crown and may be induced by a prolonged growing season (Owens and Molder 1984).

The vegetative shoot, or primary plant body laid down by the rising apical dome, is initially made up of cuticle, epidermis, cortex, primary phloem, and primary xylem (Thompson 1976). The primary phloem and primary xylem are separated by a procambium and are in vascular bundles. As development continues, the vascular cambium completely encircles the stem. The vascular cambium will then account for subsequent lateral or

secondary growth throughout the life cycle of the host (Owens and Molder 1984). The first periderm arises typically in the subepidermal layer, or occasionally in the epidermis. Sequent periderms arise in successively deeper layers beneath the first, ultimately originating from parenchyma of the secondary phloem, including ray cells (Esau 1977). The rhytidome comprises the cortex, primary and secondary phloem, and older periderms that have become separated by the last formed periderm (Trockenbrodt 1990). The seasonal development of these tissues may influence the timing of infection and the location of galls along the shoot. Internal differentiation and its effect on infection is poorly understood and is the topic of Chapter 2 in this dissertation.

1.2.2 *Host distribution and importance*

The main commercial hard pine species found in North America are *P. ponderosa*, *P. banksiana*, and *P. contorta*. Ponderosa pine is used for pattern stock, millwork, boxes, finishing, and lumber in general construction. It is found throughout North America interspersed with *Pseudotsuga menziesii* (Mirb.)Franco (Douglas-fir) and lodgepole pine (Mullins and McKnight 1981). At low elevations (<1200 m), it grows in relatively pure stands. Jack pine is used in construction for framing, sheathing, and scaffolding, and for interior woodwork. As treated lumber it is used for railway ties, fence posts, and utility poles (Collingwood and Brush 1964; Mullins and McKnight 1981). Jack pine can be found from Nova Scotia to northern Alberta, where it grows as pure stands or is sometimes mixed with aspen (Burns and Honkala 1990). Lodgepole pine is marketed with *Picea glauca* (Moench) Voss. (white spruce) and *Abies lasiocarpa* (Hook.) Nutt. (alpine fir) for use as railway ties, boxes, crates, and utility poles, and as a source of pulpwood. The tree is found in western Alberta and the interior of British Columbia and south throughout the western U.S. to Mexico (Wheeler and Critchfield 1985).

Other native hard pines found in North America include *P. muricata* D.Don. (Bishop pine), *P. radiata* Don. (Monterey pine), and *P. palustris* Mill. (long-leaf pine). Bishop pine is confined to the coast of California. It is valued for horticultural use in gardens and parks of

western Europe (Collingwood and Brush 1964). Monterey pine is native to three localities along the coast of central California. It is the most widely cultivated of all the pines, with large-scale plantations grown throughout Australia, New Zealand, and South Africa. Long-leaf pine is native to the southeastern U.S., where its wood is highly valued. (Burns and Honkala 1990).

Exotic hard pine species grown in North America include *P. sylvestris* L. (Scotch pine), *P. nigra* Arnold (Austrian pine), and *P. mugo* Turra. (Mugo pine). Scotch pine is planted for forestry purposes in eastern North America where it is the most common of all European pines. This tree has been successfully introduced in plantations, windbreaks, and ornamental plantings from North Carolina to Quebec across the Lake States to Saskatchewan (Burns and Honkala 1990). Austrian pine is a widely used exotic in North America, and in Europe it is considered an important source of commercial timber. The tree is hardy in the eastern U.S. as far north as New England and southern Ontario. Mugo pine is an introduced species from central and southern Europe (Bergdahl and French 1975). Because of its shrub-like growth, it is used in landscape and ornamental plantings in northern temperate regions (Collingwood and Brush 1964).

Hard pines will continue to be important for satisfying global timber demand. Canadian forests account for 18% of the world's forest products, as well as 50% of softwood lumber exports and 56% of newsprint exports. As the demand for these products increases, management of this forest resource will likely become more intensive. Western gall rust has been reported on many hard pine species, including *P. ponderosa*, *P. banksiana*, *P. contorta*, *P. muricata*, *P. radiata*, *P. palustris*, *P. sylvestris*, *P. nigra*, and *P. mugo* (Fig. 1.1). Several other pine hosts have been identified both in Canada and the U.S. by artificial inoculation (Boyce 1957; Hiratsuka and Powell 1976). Limiting the effects of diseases such as western gall rust should be considered in any management initiative to maximize wood production and growth.

1.3 NAME, HISTORY AND RANGE OF *ENDOCRONARTIUM HARKNESSII*

Agrios (1988, pp. 276 and 451) defined a 'rust' as a destructive plant disease characterized by many lesions on stems or leaves and caused by one of the fungi in the order *Uredinales*. The term 'rust' is derived from the rusty-brown color of the uredinial stage found in cereal rusts. Lesions are often accompanied by yellow, red, brown, or even white powdery sori. Western gall rust is characterized by bright yellow-orange sori that are most visible during sporulation. Infection caused by *E. harknessii* is easily recognized by the large globose woody galls on susceptible hosts (Hiratsuka and Maruyama 1991; Sinclair *et al.* 1987).

Rusts are generally considered morphologically primitive in their evolution, probably originating on simple hosts such as mosses and ferns during the Carboniferous Period. *Endocronartium harknessii* likely evolved from a host-alternating (heteroecious), long-cycled rust (Crane *et al.* 1995; Vogler and Bruns 1996; Vogler *et al.* 1996). Others have proposed that its differentiation occurred 30 million years ago based on present host-rust affinities and host phylogenetic histories (Millar and Kinloch 1991). Western gall rust was first reported in 1876 by H. W. Harkness from a collection found on *P. ponderosa* near Colfax, California (Harkness 1884). In 1926, H. H. York coined the name 'Woodgate rust' for a collection he made near Woodgate, New York. York's observations led him to conclude that the rust had been present since 1895 on some of the oldest *P. sylvestris* plantations in North America (True 1938; York 1926). The 'Woodgate rust' and western gall rust are now considered to be synonymous (Boyce 1957; Krebill 1970).

Western gall rust is at present restricted to the continent of North America (Arthur and Cummins 1962; Ziller 1974). The fungus ranges across Canada southward through New York, the Lake States, Nebraska, and northern Mexico (Anderson and French 1965; Peterson and Jewell 1968; Ziller 1974). *Endocronartium harknessii* has a wide host range, on five subsections of pine (Fig.1.1) and, compared to other stem rusts, a broad geographic distribution, from the Pacific to the Atlantic coasts and from sea level to over 3500 m

elevation (Millar and Kinloch 1991; Peterson 1973). Though restricted to North America, the range of *E. harknessii* is expected to expand with more widespread plantings of susceptible hard pines in non-indigenous settings. Further, pine breeding programs involving interspecific hybrids may create bridges for new genetic combinations of native rusts (Millar and Kinloch 1991).

1.4 IMPORTANCE

Endocronartium harknessii is an important pathogen because spores produced on pines directly infect pines without having to first infect an alternate host (autoecious). In naturally occurring forests, mortality from western gall rust is low, though local epidemics have been reported (Peterson 1960). In the western U.S., infections caused by *E. harknessii* occur mainly in 'wave years' and these times of high infection can ultimately cause considerable loss (Peterson 1971). Infections can lead to either branch or main stem galls. Branch galls occur at a higher frequency, resulting in irregular and altered tree form. The impact of branch galls on tree growth is negligible (Gross 1983; Hiratsuka *et al.* 1995; van der Kamp *et al.* 1994). Galls on main stems cause structural defects, predisposing infected trees to breakage during heavy snow and high winds. Alternatively, the host may be killed before stand rotation by the invasion of galls by secondary organisms (Byler *et al.* 1972*a*; Gross and Myren 1994; Sinclair *et al.* 1987). *Endocronartium harknessii* may therefore be acting as a slow thinning agent in dense natural stands of lodgepole pine (van der Kamp and Hawksworth 1985; van der Kamp 1988*a*).

Western gall rust is significant within intensively managed stands such as Christmas tree plantations, tree farms, and forest tree nurseries. Pines in landscape and Christmas tree operations may lose value because of tree stunting, witches'-brooms, and tip dieback. In New Brunswick, a planting of Scotch pine Christmas trees had to be abandoned due to deformities from high levels of infection (Forbes *et al.* 1972). In a single Christmas tree farm in Alberta 63% of the 6- to 12-year-old trees were infected with an average of 27 galls per tree (Powell and Hiratsuka 1973). Scotch pine plantations in Pennsylvania reportedly had infection

levels of 60-75%, making the tree operation economically impractical (Merrill 1972; Merrill and Kistler 1976).

Main stem galls on nursery seedlings can cause the stock to be unusable and can kill infected seedlings within a few years (Hiratsuka and Powell 1976; Peterson and Walla 1986). Mortality of infected seedlings results in lower stocking densities, reducing the effectiveness of regeneration initiatives in logged areas. Further, main stem infections often go unnoticed because gall formation can take up to 2 years to develop (Gross and Myren 1994; Hiratsuka *et al.* 1995). Once established, infected nursery stock competes with healthy seedlings within the stand. Carlson (1969) reported 3% infection on jack pine nursery stock grown near a heavily infected native stand in Manitoba. Similar infection on lodgepole pine nursery stock has also been reported for British Columbia (Doidge *et al.* 1991; Molnar *et al.* 1970).

Introduction of nonendemic rust fungi has had serious consequences. For example, the exotic rust *Cronartium ribicola* J. C. Fischer ex Rabh. has severely limited the use of *P. strobus* L. (eastern white pine) and *P. monticola* Dougl. (western white pine) as commercial species in North America (Ziller 1963; 1974). The movement of white pine to Japan has had similar repercussions (Yokota and Hama 1981). Similarly, recent establishment of Canadian provenances of *P. contorta* in Scandinavia and the introduction of North American *P. radiata* to Australia, New Zealand, and South Africa may result in severe losses should *E. harknessii* become established (Martinsson 1980; Old 1981; Old *et al.* 1985; Old *et al.* 1986; Parmeter and Newhook 1967; Scott 1960).

1.5 DISEASE SYMPTOMS

Western gall rust is characterized by globose to pear-shaped galls and to a lesser extent by hip cankers on susceptible hosts (Gross and Myren 1994; Sinclair *et al.* 1987). Cankers are likely the result of branch galls developing adjacent to main stem galls. Symptoms on branches and stems include stunting, witches'-brooms, and tip or leader dieback. Immature galls may be spindle-shaped rather than globose, causing confusion with another spindle-forming rust caused by *Cronartium comandrae* Pk. (Hiratsuka *et al.* 1995).

Needles developing beyond the center of infection frequently become chlorotic to red before the death of the branch or stem. Substandard tree form and branch dwarfing then result in response to leader death. Cone infections have been reported to occur on *P. muricata* and *P. contorta* and presumably result in the formation of main stem galls (Byler and Platt 1972).

In late May to early July, powdery yellow-orange spores known as 'aecidoiod' teliospores (spores having morphological characters of aeciospores yet functioning as teliospores) are produced and released from ruptured aecia on the gall surface (Hiratsuka 1969; Hiratsuka and Maruyama 1991). Sporulation begins 8 months to 2 years after the initial infection and continues perennially until gall death. Infection takes place on succulent tissues during the period of pine shoot elongation. First-year symptoms are typically inconspicuous and galls do not begin to form and swell until the second year after infection. True (1938) observed orange to brown discoloration of diseased tissue, which he assumed to be an early symptom of the disease. Red pigmentation develops in epidermal cells of *P. contorta* seedlings 14-28 days after infection with *E. harknessii*, but is not always a reliable indicator of gall formation (Allen *et al.* 1990*a* and 1990*b*; Kojwang and van der Kamp 1992). Early symptom development has been extensively studied by a number of researchers (Allen *et al.* 1990*a* and 1990*b*; Allen and Hiratsuka 1985; Burnes *et al.* 1988; Hiratsuka and Maruyama 1983; Hoff 1986; Hopkin and Reid 1988*a*; Hopkin *et al.* 1988; Hopkin *et al.* 1989; Kojwang and van der Kamp 1992).

After initial infection, swelling occurs, with branch galls enlarging as much as 1-10 cm, and main stem galls reaching diameters of 20-30 cm (Sinclair *et al.* 1987). Gall mortality is often associated with squirrel feeding or invasion and inactivation by secondary organisms (Hedgcock and Hunt 1920; Powell 1982; Powell *et al.* 1972). Partial or complete necrosis of the gall tissue results. Damaged areas fail to sporulate and branch or main stem death ultimately may occur.

1.6 LIFE HISTORY

Western gall rust has a reduced and simplified life cycle characterized by direct pine-to-pine infection (Hiratsuka and Powell 1976; Ziller 1974). This differs from most other pine stem rusts such as *Cronartium ribicola* and *C. comandrae*, which are host alternating and have a full complement of five separate spore stages. Wind-disseminated teliospores of *E. harknessii* land on developing pine shoots, and under cool humid conditions, germinate to produce germ tubes (Powell and Morf 1966). The germ tubes can form up to three side branches that function as basidia, directly penetrating the cuticle and epidermis. This is in contrast to indirect penetration via stomata by *C. ribicola* and *C. comandrae* (Bergdahl and French 1985; Hopkin *et al.* 1988; Patton and Johnson 1970). Infection can begin as early as May or as late as the first part of July (Ziller 1974). However, this may be subject to seasonal variation and successful infection may occur only during a 10-day period within these months (Y. Hiratsuka, personal communication).

After successful penetration of the host, an intracellular infection structure is formed from which primary hyphae grow intercellularly into the epidermis and cortex. Haustoria form in adjacent cells (Hopkin and Reid 1988*a* and 1988*b*; Hopkin *et al.* 1989). Haploid hyphae continue to proliferate throughout the cortex, reaching the vascular cambium prior to host dormancy in the first year. The cambium is invaded from the phloem and cortex in an inward fashion rather than by vertical or peripheral spread of hyphae (True 1938). Gall formation is initiated by exogenous stimulation of the cambium and ray initials, causing an increased production of ray parenchyma (Jewell *et al.* 1962; Peterson 1960; Zalasky 1976). The predominant host reaction to hyphal invasion is increased cell division (hyperplasia). Subsequent proliferation of hyphae into the developing cortex, phloem, and vascular cambium accounts for increased annual growth increments until the death of the gall. During the first 2 years the gall continues to enlarge, but does not sporulate until the third year.

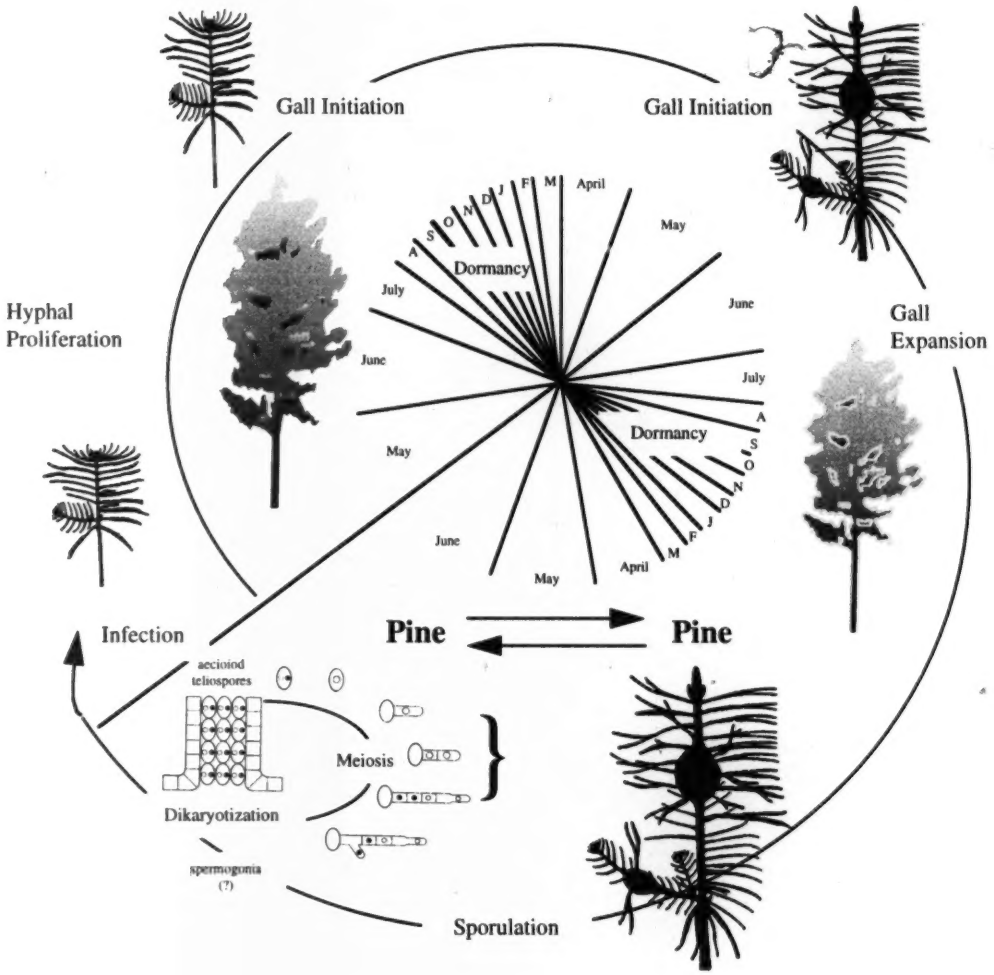
Spermogonia have been observed early in the spring and produce a sticky ooze which emerges from infected bark (Crane *et al.* 1995; Walla *et al.* 1991*a*). The spermogonia are

considered non-functional, since there is no consistent association with developing aecia as in other stem rusts. They are believed to be vestiges of a sexual stage lost during the recent evolution of the fungus (Crane *et al.* 1995; Vogler and Bruns 1996; Vogler *et al.* 1996). Consequently, it is doubtful that spermongonia play a major role in dikaryotization, since they are rarely produced in nature. Dikaryotization of the haploid mycelium takes place in the outer cortex just beneath the first periderm. The dikaryotic mycelium produces teliospore initials early in the spring of the third year. The first few cell layers produced by the initials make up an outer enveloping membrane of the aecium known as a peridium. The aecia rupture from the increased production of spores beneath the peridia, leaving behind a blister and exposing the abundant yellow-orange 'aecidioid' teliospores. The spores are then passively liberated to infect newly emerging host tissue, thereby completing the life cycle. The exact stimulus of dikaryotization is poorly understood, though it is likely triggered by the annual initiation of host sap flow and other environmental cues. The life cycle of *E. harknessii* is shown in Fig. 1.2.

1.7 CLASSIFICATION

The rusts as a group are included in the monophyletic kingdom *Fungi*, phylum *Basidiomycota*, order *Uredinales*. Family designations within *Uredinales* are controversial (Alexopoulos *et al.* 1995). The traditional families *Puccinaceae* and *Melampsoraceae* have been supported on the basis of teliospore morphology, the former having pedicellate teliospores, whereas the latter produce crusts or columns. Morphology of aecia and aeciospores, peridial cells, uredinia and urediniospores, and basidia and basidiospores, as well as known host relationships have all been used to classify hard pine stem rusts (Arthur and Cummins 1962; Peterson and Jewell 1968; Rhoads *et al.* 1918; Ziller 1974). Hiratsuka and Cummins (1963) suggested that telial states were overemphasized in earlier classification schemes and proposed that greater emphasis be given to spermogonia. They delineated 14 families (Cummins and Hiratsuka 1983).

Figure 1.2. Life History of *Endocronartium harknessii* (J. P. Moore) Y. Hiratsuka..



The life cycle of *E. harknessii* extends over 26 months, from initial infection to first sporulation. Spiral represents gall development and internal pathogenesis. The center of the spiral is divided into months (Hiratsuka 1973).

The genus *Endocronartium* was erected to accommodate the endocyclic North American *E. harknessii* and European *E. pini* (Pers.) Y. Hiratsuka (Hiratsuka 1969). Since that time, two new species have been added to the genus, *E. sahoanum* Imazu & Kakishima and *E. yamabense* (Saho & Takahashi) Paclt., both occurring in Japan (Imazu *et al.* 1989; Imazu *et al.* 1991*a* and 1991*b*). In addition, a new *Endocronartium* species has been reported from central China (Jing *et al.* 1995). Much debate surrounds the nomenclatural interpretation and the endocyclic nature of the genus (Epstein and Buurlage 1988; Laundon 1976; Vogler *et al.* 1991; Vogler *et al.* 1996). Hiratsuka has since reaffirmed his contention that karyogamy and meiosis are occurring within the spore and germ tube, respectively. Based on these findings, preservation of the genus is valid (Hiratsuka 1973*a*; 1974). Observation of synaptonemal complexes in prophase I nuclei in the germ tubes has been confirmed (Y. Hiratsuka, personal communication) providing convincing evidence of meiosis (Hiratsuka 1991*a*).

Pine stem rusts have been divided into three genera, *Cronartium*, *Endocronartium*, and the form-genus *Peridermium*. At present, *Cronartium* has 16 validly described species, *Endocronartium* has five, and the form-genus *Peridermium* contains 17 (Hiratsuka 1995). Pine stem rusts and their phylogenetic relationships have been extensively investigated. As molecular techniques are further applied, important evidence for determining relationships within this group should be forthcoming (Hiratsuka 1995; Powers *et al.* 1989; Sun *et al.* 1995*a*; 1995*b*; Tuskan and Walla 1989; Tuskan *et al.* 1990; Tuskan *et al.* 1991; Vogler 1995; Vogler and Bruns 1993; Vogler *et al.* 1996). Isozyme analysis has confirmed a strong correlation between *E. harknessii* and *Cronartium quercuum* (Berk.) Miyabe ex Shirai., the macrocyclic heteroecious causal agent of pine-oak rust. Two forms of banding patterns designated zymodeme I and zymodeme II, have been consistently observed within the genus *Endocronartium* (Vogler *et al.* 1991). It has been suggested that zymodeme I is a recent immigrant to the west, probably since the last ice age, whereas zymodeme II existed during glaciation and is presumably indigenous to the western U.S. Albino races commonly

designated as *E. harknessii* may have no relationship at all to this genus and may in fact be new species worthy of further investigation (Christenson 1969; Vogler *et al.* 1996; Walla *et al.* 1991*b*).

1.8 EPIDEMIOLOGY

Endocronartium harknessii epidemics are rare events. Outbreaks result when an intricate balance is disrupted between host, pathogen, and environment. Under optimal conditions, a burst of infection can take place in a single season. This is known as a 'wave year' of infection (Peterson 1971; van der Kamp 1988*a*). Following a wave year, there may be many years when infection tapers off as the rust infections die out (Peterson and Jewell 1968). In three lodgepole pine plantations in British Columbia, a wave year was reported to have occurred in 1990, with resultant galls detected in 1992 and spore production in 1993 (van der Kamp 1994; van der Kamp *et al.* 1995). The frequency and intensity of infection depends on regional stand dynamics (i.e. slope, aspect, temperature, relative humidity, wind speed), as well as host distribution. In west-central Alberta, *E. harknessii* incidence was higher on east-facing slopes than either north- or south-facing slopes in lodgepole pine stands older than 15 years (Bella and Navratil 1988).

Diurnal periodicity of *E. harknessii* spore release has been documented (Chang *et al.* 1989; Peterson 1973). Spore release is correlated with light, wind speed, and relative humidity. A build-up of spores occurs during the evening, possibly due to increased water potential in the bark; this is followed by passive spore release during the day (Blenis *et al.* 1993*b*). Rain also may trigger the release, causing spores to clump together, dry out, and subsequently be disseminated by wind (Chang *et al.* 1989). Under simulated conditions, survival of *E. harknessii* spores was favored by low relative humidity (39%) and cool temperatures (6-15°C), suggesting a strong potential for long distance dispersal (Chang and Blenis 1989). This, coupled with variable pathogen virulence, may be particularly important if trees are selected for resistance to local populations of *E. harknessii.* Infections may be limited by spore production, spore viability, and host development. However, the relative

importance of such factors is poorly understood and more information is needed for developing site hazard ratings.

1.9 CONTROL

As with most plant diseases, there are few control methods capable of entirely eliminating the pathogen. Different disease management practices have different advantages and disadvantages. Probably the most important criterion determining the use of these strategies is their economic feasibility. Strategies such as breeding for host resistance, controlling planting density, spacing and thinning, identifying low-hazard sites, and chemical and biological control may have practical application in intensively managed pine plantations. To date, none of these have proven totally effective for controlling western gall rust, though economic losses to this disease may be minimized under certain conditions (Hiratsuka and Powell 1976). An integrated approach combined with an increase in the stocking levels to offset mortality in high risk areas may afford the best management for this disease (Hills *et al.* 1994; van der Kamp 1994).

1.9.1 ***Disease resistance***

Resistance is conditioned by several internal and external factors that limit infection (Agrios 1988). Pine trees may have immunity to the pathogen (non-host resistance), specific genes for resistance (true resistance), or for various reasons, simply escape or tolerate infection (apparent resistance). Anatomical changes that take place during seasonal growth and maturation can also act as barriers to infection (Kojwang and van der Kamp 1992; Power *et al.* 1994; Zagory and Libby 1985). Van der Kamp and Tait (1990) have demonstrated stable susceptibility in a natural pine population in spite of the pathogen cycling at least 25 generations for each host generation. Natural infection levels rarely reach epidemic proportions even when abundant infection courts and high levels of inoculum are present in the stand. The sporadic arrival of inoculum at the infection courts and lack of favorable climatic conditions are likely non-genetic factors which limit infection (van der Kamp 1988*b*;1993). There is strong evidence to suggest that some combination of the host

resistance mechanisms described above decrease incidence of western gall rust.

Today, most resistance studies of pine focus on the quantitative and qualitative nature of true resistance (Blenis *et al.* 1993*a*; Hoff 1991*a*; 1991*b*; Hoff and Minggao 1994; Klein *et al.* 1991; Yanchuk *et al.* 1988; van der Kamp 1991*a*; 1991*b*). In the concept of the 'gene-for-gene' theory, Flor (1955) postulated that rust race distinction is possible based on susceptible host response. A plant line will be resistant only if a race of rust has at least one gene for avirulence and the host at least one matching gene for resistance. A plant line will be susceptible if the host has no resistance genes, resistance is recessive in the host, or the rust race has virulence genes to counter the resistance genes found in that host. These reactions enable researchers to assess host susceptibility to a range of specified races. This type of work may be useful for the gall rust pathosystem (Allen *et al.* 1990*b*; Burns and Blanchette 1991; Hoff 1991*b*; Kinloch 1982). There has been some suggestion, however, that reaction type rating is poorly correlated with gall formation and may inadequately reflect resistant families or individuals in this pathosystem (Allen *et al.* 1990*b*; Kojwang 1994; Kojwang and van der Kamp 1991; Kojwang and van der Kamp 1992). Furthermore, counting the number of galls may ignore other non-genetic factors that account for a large portion of variation in natural infection levels among trees in the field (van der Kamp and Tait 1990; van der Kamp 1993).

Operationally, controlled inoculations provide the most useful means for screening many pine families for resistance to *E. harknessii* (Burnes *et al.* 1989; Lundquist *et al.* 1991). If pine families are sufficiently challenged with high levels of spores collected from the area in which they grew, then families which show high resistance are likely to be resistant in the field (Blenis and Hiratsuka 1986; Blenis and Pinnell 1988; Kojwang 1994; van der Kamp 1989). Improvements to artificial inoculation techniques such as precise spore concentrations (Blenis and Pinnell 1988), the 'torn leaf' method (Myrholm and Hiratsuka 1993), and the use of marker-assisted selection (MAS) for locating resistance genes (Hua 1996; Yazdani *et al.* 1995) may give reliable numbers of truly resistant off-spring. Long-term field testing

of these resistant progeny is needed in each geographical region before large plantings are implemented.

1.9.2 *Silviculture*

For most commercial pine species, justification for silvicultural control of western gall rust will remain one of financial discretion (Peterson and Jewell 1968). The amount allocated will depend on the level of management planned for a pine plantation or natural stand, and will increase when more value is assigned on a per tree basis such as in Christmas tree plantations (Boyce 1961; Kistler and Merrill 1978). Measures such as precommercial thinning, manipulating stocking density, and site hazard rating may produce adequate economic returns to justify their cost. However, successful control will depend on site specific management goals and objectives. Recent studies have focused on each of these stand tending methods and much is known about their efficacy (Blenis and Duncan 1997; Bella 1985*a* and 1985*b*; Bella and Navratil 1988). From such investigations, better prediction and application of appropriate control measures can be obtained (Blenis and Duncan 1997; Hills *et al.* 1994; van der Kamp 1994).

Precommercial thinning operations are routinely done on naturally regenerated lodgepole pine in Alberta, where ideal stocking is 2500 stems per hectare (Bella 1985*a*; Bella and Navratil 1988). The age at which these stands are thinned may be of particular importance, since stem infections are known to reach maximum severity by 17-20 years. During a precommercial thin, emphasis should be placed on removing trees that have main stem galls or a high incidence of branch galls (Blenis and Duncan 1997; Hills *et al.* 1994). However, this might lead to non-uniform spacing due to the random distribution of the disease in the stand (van der Kamp 1994; van der Kamp and Spence 1987). In such cases, leaving more trees on site may be advisable to compensate for any additional increase in tree mortality.

In northwestern Ontario, incidence of *E. harknessii* increased at 1.5-m spacing and decreased significantly at 3-m spacing (Hills *et al.* 1994). However, in British Columbia there

was no relationship between stand density and infection frequency (van der Kamp 1994). Because precommercial thinning is an important silvicultural tool, it is important that the effects of *E. harknessii* at various densities be further evaluated. Van der Kamp (1994) outlined three possible lodgepole pine stocking densities for the interior of British Columbia. Minimum stocking would barely capture full site productivity and would not allow for additional tree death. Maximum stocking is that level which would not stagnate the stand but instead provide optimal diameter growth. Optimum stocking would be somewhat greater than minimum stocking. While these densities are largely stand specific, targeting the optimum and accepting some loss could be the most cost efficient strategy. Prescribing a commercial thin 20-30 years before final harvest would allow the recovery of lost revenues, as infected trees could be used for pulpwood. This would have the additional benefit of releasing the remaining trees, maximizing growth potential (Bella 1985*a*; Peterson and Jewell 1968).

1.9.3 *Cultural and chemical control*

On ornamental trees or high value Christmas tree plantings, it may be advisable to rogue trees or prune branch galls on heavily infected individuals, thus reducing the inoculum potential in the stand. Care should be exercised in locating such plantings and it is recommended that trees with galls be removed for a distance of 300 m around nurseries (Boyce 1961; Hiratsuka *et al.* 1995). Chemical control of *E. harknessii* has been investigated and could be used as a protective measure for high value trees (Hinds and Peterson 1966; Huber 1980; Merill and Kistler 1976). Benodanil applied at the recommended rate reduced infection, higher levels, or a second application, may be necessary for adequate control (Wenner and Merrill 1987). Even the antimicrobial effects of smoke from fire (natural or prescribed) have been reported to confer some protection (Parmeter and Uhrenholdt 1975). Such controls show promise but are costly and difficult to administer on a large scale.

1.9.4 *Biological control*

The use of natural biological agents against disease is appealing if it can be

done using simple procedures at a low cost and in the absence of residual effects to the environment (Wicker 1981). Historically, the use of these agents to control disease has been postulated largely on the basis of field observations of occurrence of organisms antagonistic to pathogens. Research efforts have advanced from mycological curiosity to understanding the mechanisms behind biocontrol and ultimately their efficacy under controlled environments. Andrews (1990) concluded that despite decades of biocontrol research only two major discoveries have been made: *Agrobacterium radiobacter* strain 84 for control of crown gall caused by *Agrobacterium tumefaciens* (Kerr and Tate 1984), and the application of *Peniophora gigantea* to pine stumps for control of *Heterobasidion annosum* (Rishbeth 1963). While there is no shortage of potential biological control agents, many of these organisms have not passed important tests for their ultimate use in commercial application (Cook 1996). Given the limited number of success stories, it is easy to understand why foresters have not been attracted to biological control. However, as demand for wood products increases, this type of control strategy may become more attractive. Biological control should not be viewed as a single solution; rather it must be integrated with several management schemes to reduce losses from disease (Andrews 1992).

Many fungi, insects, slugs, and rodents are known to play a role in the natural limitation of pine stem rusts (Bergdahl and French 1978; Byler *et al.* 1972*a* and 1972*b*; Hedgcock and Hunt 1920; Hiratsuka *et al.* 1979; Hunt 1978; Kuhlman 1981*a* and 1981*b*; Parsons 1967; Powell 1971*a* and 1971*b*; Salt and Roth 1980; Wong 1972). Secondary fungi in particular have shown a close association with *E. harknessii* and may be responsible for mortality of the gall (Byler *et al.* 1972*a* and 1972*b*; Sutton 1973; Tsuneda and Hiratsuka 1981*b*; Tsuneda and Hiratsuka 1982). Table 1.1 summarizes the known fungal inhabitants associated with *E. harknessii* on various pine hosts. Many of these fungi have been screened as potential candidates for biological control. The evidence reported for potential control is convincing; however, actual control of pine stem rusts has yet to be demonstrated in the field (Hiratsuka and Powell 1976; Tsuneda and Hiratsuka 1981*a*). Success of these agents will

Table 1.1. Secondary fungal inhabitants of *E. harknessii* found on various pine hosts. This list is comprised from personal observation and the following literature citations: Byler *et al.* 1972*a* and 1972*b*; Hiratsuka *et al.* 1979; Powell and Morf 1965; Sutton 1973; Tsuneda and Hiratsuka1980; Wollenweber 1934.

Host	Secondary fungi[f]
P. contorta	***Fusarium bactridiodes* Woll.**
	***Gibberella lateritium* (Nees.) Snyder & Hansen**
	***Diplodia pinea* (Desm.) Kicks**
	***Nectria fuckeliana* Booth**
	Penicillium sp.
	Scytalidium uredinicola* Kuhl. *et al.
	***Cladosporium gallicola* Sutton**
	***Monocillium nordinii* (Bourchier)W.Gams**
	***Tuberculina maxima* Rost.**
	Scopinella gallicola A. Tsuneda & Y. Hiratsuka
	Aureobasidium sp.
P. radiata	*Gibberella lateritium*
	Diplodia pinea
	Nectria fuckeliana
	Pullularia sp.
	Penicillium uredineicolum Hulea
	Botrytis sp.
	Phomopsis sp.
	Alternaria sp.
	Camerosporium sp.
	Cladosporium aecidiicolum
	Plenodomona sp.
P. muricata	*Gibberella lateritium*
	Diplodia pinea
	Nectria fuckeliana

f Boldface indicates fungi considered for microbial biocontrol of *Endocronartium harknessii.*

require a basic understanding of species interactions and the factors influencing fungal communities on galls. Most of what is now known centers around the various modes of parasitism (i.e. necrotrophic or biotrophic) and the chemical nature of inhibition of target pathogens by specific fungal metabolites.

In western Canada, *Monocillium nordinii* (Bourchier) W. Gams., *Cladosporium gallicola* Sutton, and *Scytalidium uredinicola* Kuhlman *et al.* have all been found to be mycoparasites of *E. harknessii* (Hiratsuka 1979; Tsuneda and Hiratsuka 1981*a*). When fresh teliospores of *E. harknessii* were challenged with a suspension of *M. nordinii*, hyphae grew between the surface wart layers of spores, forming a swollen appressorium-like body at points of contact (Tsuneda and Hiratsuka 1980). Only after prolonged interaction did the mycoparasite directly penetrate the rust teliospores. Under field conditions, *M. nordinii* appeared less evident and more unevenly distributed than *C. gallicola* or *S. uredinicola*. In spite of its prolific vertical growth, horizontal spread of this fungus is restricted. Inactivation of the gall may therefore be incomplete. Nevertheless, *M. nordinii* has been reported to produce antifungal compounds such as mononden and monocillin I, which are antagonistic against a wide array of fungi (Ayer and Peña-Rodriquez 1987; Ayer *et al.* 1980).

Cladosporium gallicola parasitizes *E. harknessii* by contact or direct penetration, causing the surface cell wall layer of teliospores to disintegrate (Tsuneda and Hiratsuka 1979). The mode of action is presumably enzymatic or some other chemical-induced breakdown of host tissues. Under moist conditions, this fungus is known to sporulate profusely and exhibit rapid hyphal growth over the surface of infected galls. Culture filtrate of *C. gallicola* did not have an obvious impact on *E. harknessii*, and it appears hyphal contact with host spores is necessary for the mycoparasitic process (Tsuneda and Hiratsuka 1979). In the field, dark olive-green colonies can be observed on sporulating galls. Subsequent identification can be masked by other fungal inhabitants and may be determined only through microscopic and cultural evaluation. *Cladosporium gallicola* and *C. aecidiicolum* Thuem. are both restricted to exposed sori and do not invade the wood of living galls (Byler *et al.* 1972*b*; Tsuneda and

Hiratsuka 1981*a*). The timing of *C. gallicola* and its relationship to *S. uredinicola* is addressed in Chapter 4 of this dissertation.

1.10 *SCYTALIDIUM UREDINICOLA*

Kuhlman and co-workers (1976) originally discovered *S. uredinicola* on *Cronartium fusiforme* Hedgc. & Hunt ex Cumm. aecia on loblolly (*P. taeda* L.) and slash pine (*P. elliottii* Engelm. var. *elliottii*) in the southeastern U.S. Its presence reduced spore production on galls by 20% (Kuhlman 1981*a* and 1981*b*). *Scytalidium uredinicola* was later identified from the surface of active *E. harknessii* sori on jack and lodgepole pine in Alberta, Canada (Hiratsuka *et al.* 1979). Like *C. gallicola*, *S. uredinicola* is capable of disintegrating teliospores of *E. harknessii* (Chakravarty and Hiratsuka 1995; Tsuneda *et al.* 1980). Parasitism is likely accompanied by the diffusion of chemicals such as enzymes in advance of the mycoparasite's growth. When teliospores were challenged with a spore suspension of *S. uredinicola*, degradation of the spore wall occurred within 48-72 h, without penetration (Tsuneda *et al.* 1980). The mycoparasite also adversely affected the basal cell region of active sori and rust hyphae deep within the wood tissue. The combined inactivation of spores and immature spore layers is more effective than the action of either *M. nordinii* or *C. gallicola*; thus *S. uredinicola* is the most logical candidate for use in control of *E. harknessii* (Hiratsuka 1991*b*; Hiratsuka *et al.* 1987).

Fairbairn *et al.* (1983) was able to isolate from *S. uredinicola* a chloroform-extractable material with a molecular weight of less than 10 000 that inhibited germination of *E. harknessii*. Further analysis of *S. uredinicola* grown on 2% malt extract broth revealed an inhibitory compound, 3-hydroxy-2-4H-pyranone, or maltol, which caused a 50% reduction in the germination of *E. harknessii* at 50 µg/ml (Cunningham and Pickard 1985). Maltol applied to lodgepole pine seedlings produced a significant reduction in *E. harknessii* infection (Blenis *et al.* 1988). In addition to maltol, chemosystematic studies in the genus *Scytalidium* have produced scytalidin, scytalone, deoxyscytalidin, and lignicol, all of which have antagonistic effects on wood decay fungi (Ayer *et al.* 1993; Klingström and Johansson

1972; Stillwell *et al.* 1973). However, these specific metabolites have not been isolated from *S. uredinicola.*

Scytalidium uredinicola is distinguished by its gray-green appearance on the otherwise bright yellow-orange sori of *E. harknessii.* The hyphae of *S. uredinicola* break up arthrothallically into cylindrical conidia, which initially measure 1.5-2 X 2-4 μm. As the conidia mature, they swell, becoming obvoidal to subglobose and attaining sizes of 3-4(-5.6) X (1.6-)3-4 μm. This sequence of development differs significantly from other described species of *Scytalidium* such as *S. circinatum* Sigler & Wang, *S. lignicola* (Pesante), and *S. album* Beyer & Klingström (Sigler and Carmichael 1976; Sigler and Wang 1990). In culture, growth of *S. uredinicola* was best on malt extract and yeast agar at 25°C (Pickard *et al.* 1983; Kuhlman *et al.* 1976). Fairbairn and co-workers (1983) found maximum yields of mycelial dry weight occurred when the fungus was cultured on liquid phytone-yeast extract-malt extract (PYME) and malt extract media. The growth of *S. uredinicola* can also be enhanced using carrot agar (C. Myrholm, personal communication).

Successful use of *S. uredinicola* metabolites for control in nature may be hindered by environmental conditions not comparable to the laboratory. This, along with the small amounts of inhibitory compounds produced, may mean large quantities of fungal isolates will be needed to recover sufficient control product. Even if successful, administration of these compounds in the field must be closely monitored in long term field trials to determine their chemical stability in fluctuating environments. In addition, their impact on beneficial microflora may limit natural controls presently acting upon *E. harknessii.* More research is therefore needed on the synecology of gall-inhabiting fungi and the conditions in nature under which *S. uredinicola* becomes most active. Nonetheless, advances made in these areas may one day lead to patents of natural products for control that are target-specific and less detrimental to the environment than traditional fungicides (Tsuneda and Hiratsuka 1981*a*).

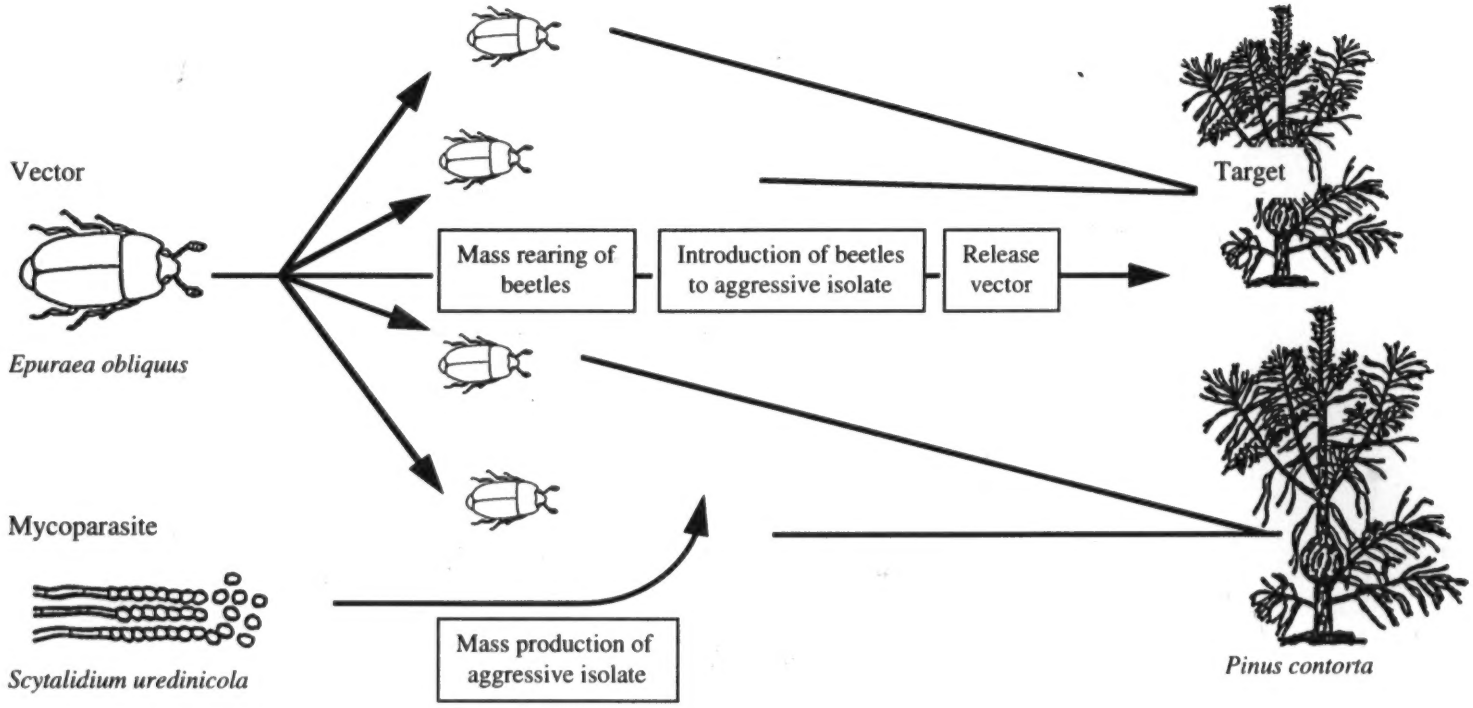
As mentioned, several insects and free-moving organisms are known to feed on pine stem rust spores and infected tissues (Hunt 1978; Nelson 1982; Powell 1971*a*; 1971*b*. There

are strong indications that *Epuraea obliquus* Haatch (Coleoptera: Nitidulidae) feeds upon *E. harknessii* spores and serves as the primary vector of *S. uredinicola* in nature (Currie 1995; Currie *et al.* 1995; Currie and Hiratsuka 1996). The beetles are therefore vital to initial infection and subsequent dissemination of *S. uredinicola* throughout the stand. Hiratsuka (1991*b*) proposed a biological control strategy that would use this target-specific feeding relationship. Aggressive isolates of *S. uredinicola* would be placed on this vector, which would be released into infected stands of lodgepole pine. This strategy is summarized in Fig. 1.3. Before such a strategy can be successfully implemented, fundamental questions concerning distribution, life history, and persistence of *S. uredinicola* in the suscept's environment must be addressed. For example, although the mode of dispersion has been established, seasonal development of the mycoparasite is poorly understood. Because of the complexity of this biological system, efforts have recently been undertaken to model the impact of hyperparasites on western gall rust (van der Kamp and Blenis 1996).

1.11 IMPLICATIONS

Western gall rust will continue to pose a threat to North American hard pines. The potential for spread to other areas of the world should intensify efforts to minimize this disease. While there are known control measures, none to date have proven totally effective. More information is needed on factors limiting infection. Further defining when infection occurs during the growing season would improve the forecasting of *E. harknessii* epidemics and establish the time frame under which natural control agents must operate. Biological control strategies using aggressive mycoparasites are appealing since control is target specific and occurring naturally in the pathosystem. However, before such strategies can be implemented, careful study of their biology and localized development are required to ensure success in the field. Modeling the impact of mycoparasites on western gall rust may provide a basis for predicting the long term effects of these organisms. However, more field investigation is required to verify the assumptions and improve the accuracy of such models.

FIGURE 1.3. Proposed biological control strategy for western gall rust (Hiratsuka 1991*b*). This strategy seeks to exploit the target-specific feeding pattern of *E. obliquus* by mass rearing the beetles then exposing these vectors to an aggressive isolate of *S. uredinicola*. Beetles are then released to galls on lodgepole pine.



1.12 OBJECTIVES OF RESEARCH

i. Describe the relative importance of spore production, spore viability of *E. harknessii*, and shoot elongation of *P. contorta* in limiting infection throughout the growing season.

ii. Document the incidence of *S. uredinicola* on western gall rust in west-central Alberta.

iii. Demonstrate the effects of gall age, gall size, and rust severity on the incidence of *S. uredinicola*.

iv. Determine the occurrence of *S. uredinicola* in external and internal tissues of the gall throughout the growing season on galls of variable age.

v. Describe the location and development of *S. uredinicola* throughout the growing season on main stem galls

vi. Examine the effect of *S. uredinicola* parasitism on the viability of gall rust spores during sporulation in the field.

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CHAPTER 2

FACTORS INFLUENCING *ENDOCRONARTIUM HARKNESSII* INFECTION ON *PINUS CONTORTA*

2.1 INTRODUCTION

Endocronartium harknessii (J.P. Moore) Y. Hiratsuka is an autoecious rust fungus that poses a threat to North American hard pine nurseries, Christmas tree plantations, and precommercially thinned stands (Bella and Navratil 1988; Blenis and Duncan 1997; Merrill and Wenner 1988; Ziller 1974). Loss results when main stem infections lead to tree death from wind breakage or invasion by other gall-associated fungi (Byler *et al.* 1972; Hiratsuka *et al.* 1995). The distribution of western gall rust is expected to increase as susceptible pine hosts are planted to non-indigenous settings (Martinsson 1980; Old *et al.* 1985; Parmeter and Newhook 1967).

Although there are silvicultural practices that may reduce the impact of western gall rust (Blenis and Duncan 1997; van der Kamp 1994), there are only a few practical, direct methods for control. Chemical control has been used on high value trees (Kistler and Merrill 1978; Merrill and Kistler 1976; Wenner and Merrill 1987). Biological control has also been suggested, since there are several known mycoparasites of western gall rust (Byler *et al.* 1972; Tsuneda *et al.* 1980), such as *Scytalidium uredinicola* Kuhlman *et al.* (Currie and Hiratsuka 1996) that could potentially be used in managing the disease (Hiratsuka 1991). Host resistance has been widely considered, and may provide long term control in intensively managed plantations. (Allen *et al.* 1990; Kojwang 1994). However, it has been suggested that the benefits of genetic resistance may vary with extent to which the western gall rust population is regulated by mycoparasites (van der Kamp and Blenis 1996).

Regardless of whether chemical or biological control is used, it is essential to know when during the year infection occurs, so that applications of control agents are properly timed. Furthermore, when trying to determine which mycoparasites are important in regulating the western gall rust population, the focus should be on those mycoparasites that are active and abundant prior to, or during, the time when infections are common. Our understanding of the mechanisms that alter pathogen success could be improved if the period of maximum susceptibility were better defined.

It has long been known that most gall rust infections occur in the spring and summer when the pathogen is abundant and host tissues are succulent (True 1938). The periodicity and conditions under which spores are dispersed has been well documented (Peterson 1972; Chang *et al.* 1989). More recently, rust sporulation was shown to be synchronized with host development in *Pinus sylvestris* L. Christmas tree plantations in Pennsylvania (Merrill and Kistler 1976). Dissemination of spores began as needles emerged beyond the fasicle sheath and had ceased by the time the needles had grown to 75% their maximum length. Differences in disease incidence also were demonstrated in *P. contorta* Dougl. seedlings inoculated at two different maturity levels (Kojwang and van der Kamp 1992). Although these previous studies demonstrated an association between shoot age and susceptibility, the exact pattern of this association is still unclear. Furthermore, it is unknown whether spore viability remains uniformly high during the growing season or whether there are changes in germinability that are important in determining the temporal pattern of infection. Hence, the first objective of this study was to establish the role of *E. harknessii* spore production, spore viability, and *P. contorta* shoot age play in influencing gall rust infection.

Because it is difficult to maintain optimum conditions for infection in the field, inoculations in the field cannot be used to determine the relationship between shoot age and susceptibility to infection. Although controlled inoculations of potted trees in a greenhouse or growth cabinet can be used to determine temporal changes in susceptibility, it is not certain whether these patterns would be the same as for larger trees in the forest. Complete resolution of this problem is likely impossible. However, if it could be shown that patterns of shoot development were similar in greenhouse grown and forest grown trees, our confidence in the use of greenhouse grown trees to determine patterns of susceptibility would be increased. Therefore, our second objective was to compare patterns of tissue development between greenhouse grown and field grown trees at similar stages of shoot elongation.

2.2 MATERIALS AND METHODS

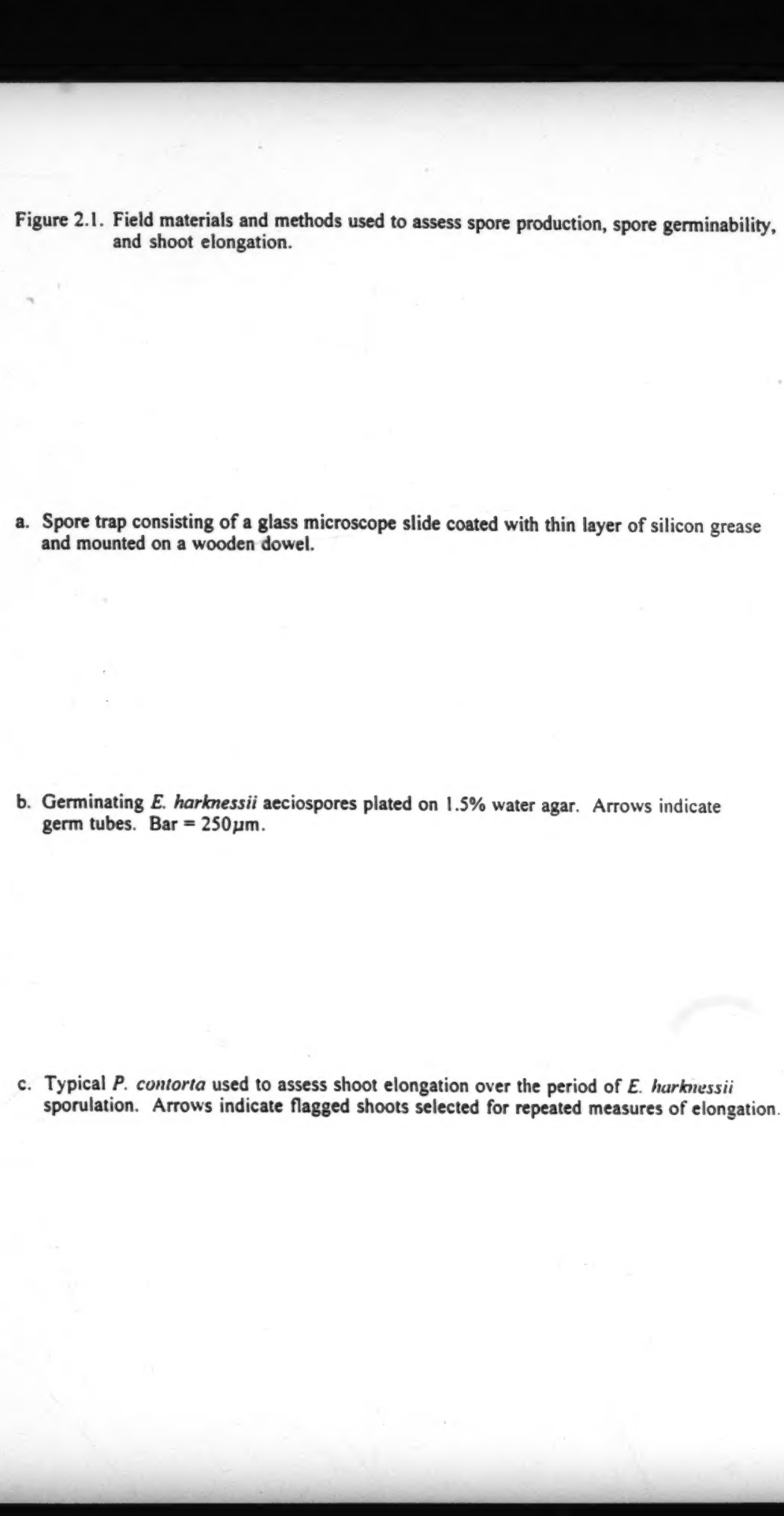
2.2.1 *Spore production, spore viability, and shoot elongation in the field*

Field studies were conducted in 1992 and 1993 in two *P. contorta* stands located near Hinton, Alberta. Site 1 was 20 years old and 21.3 hectares in size, whereas site 2 was 19 years old and 86 hectares in size. To assess spore production, spore traps consisting of glass microscope slides (25 X 150 mm) thinly coated with silicon grease, were mounted on top of wooden dowels approximately 1 m in height (Fig. 2.1a). Twenty spore traps were spaced 60 m apart along one transect per site in 1992. Ten spore traps were used per site in 1993. Two-day totals of spore production were determined from 20 May to 17 July, 1992, and 21 April to 7 July, 1993.

To determine *E. harknessii* spore viability, 10 galls with no obvious signs of hyperparasitism were collected from both stands at weekly intervals from 20 May to 29 June, 1992, and from 10 May to 1 July, 1993. Within 30 minutes of gall collection, spores were dislodged, by a short burst of air, onto the surface of 1.5% water agar plates. Germination of 100 spores per plate was assessed following 12-h incubation in darkness at 18°C (Fig. 2.1b).

Shoot length was measured weekly on eight trees per site from 20 May to 17 July, 1992, and from 21 April to 7 July, 1993, for a total of 32 trees over the 2 years. Five randomly selected shoots per tree were repeatedly measured (Fig. 2.1c) and shoot elongation at each time was defined as current shoot length expressed as a percentage of final shoot length. For each combination of year and stand, a separate second order polynomial was used to express shoot elongation as a function of sampling date. This relationship was then used as the basis for expressing spore production and viability as a function of shoot elongation. At each sampling date, an additional eight shoots were selected at random, excised from each tree, and preserved in formalin-acetic-acid (FAA) for histological staining and sectioning (Sass 1958).

Figure 2.1. Field materials and methods used to assess spore production, spore germinability, and shoot elongation.



a. Spore trap consisting of a glass microscope slide coated with thin layer of silicon grease and mounted on a wooden dowel.

b. Germinating *E. harknessii* aeciospores plated on 1.5% water agar. Arrows indicate germ tubes. Bar = 250μm.

c. Typical *P. contorta* used to assess shoot elongation over the period of *E. harknessii* sporulation. Arrows indicate flagged shoots selected for repeated measures of elongation.

a
b
c

2.2.2 *Greenhouse inoculations*

Spores were collected from four locations near Hinton, Alberta, in 1993 and 1994. Spores from several galls were combined to make single spore lots which were passed through a 45-μm sieve, placed in open 30-ml vials, kept up to 5 days at 4°C in a desiccator containing $CaCl_2$, transferred to 2-ml vials, and finally placed in liquid nitrogen for storage. Controlled inoculations were conducted in 1994 and 1995. Dormant 2-year-old *P. contorta* seedlings were obtained from the Pine Ridge Forest Nursery, located at Smoky Lake, Alberta and maintained in cold storage at -4°C. For each inoculation in 1994 and 1995, one set of 21 and 30 seedlings, respectively, was removed from cold storage each week over a 4-week period to establish a range of seedling development classes (Fig. 2.2a). The seedlings were thawed for 2 days at 4°C, planted in "Fives" Spencer / Lemaire rootrainer® trays with a mixture of lime-supplemented peat (pH 5.5), and maintained in a greenhouse under an 18-h photoperiod at 20°C. Prior to inoculation, three vials of spores, each from a different spore lot, were removed from liquid nitrogen, warmed in a 40°C water bath for 5 minutes and combined. Percentage germination was determined as above at the time of each inoculation.

Seedlings were marked with a non-toxic tree paint at the base of the developing shoots (Fig. 2.2b), and measured for initial shoot length (Fig. 2.2c). A #12 Demco® Series 140, (Demco Inc., Madison, WI) paint brush was used to apply spores over the entire developing shoot (Fig. 2.2d). Seedlings were inoculated 3 days after the last of the four sets of trees per inoculation had been removed from storage. Hence, seedlings would have been on the greenhouse bench 3, 10, 17, or 24 days at the time of inoculation. Prior to inoculation in 1994 and 1995, seedlings were arranged in trays such that each tray had three and five seedlings, respectively, in each of four different stages of development. In both years, there were four separate inoculations, consisting of six inoculated trays and one control tray for a total of 336 seedlings in 1994, and five inoculated trays and one control tray for a total of 480 seedlings in 1995. For each tree, shoot elongation at the time of inoculation was defined as shoot length at that time expressed as a percentage of final shoot

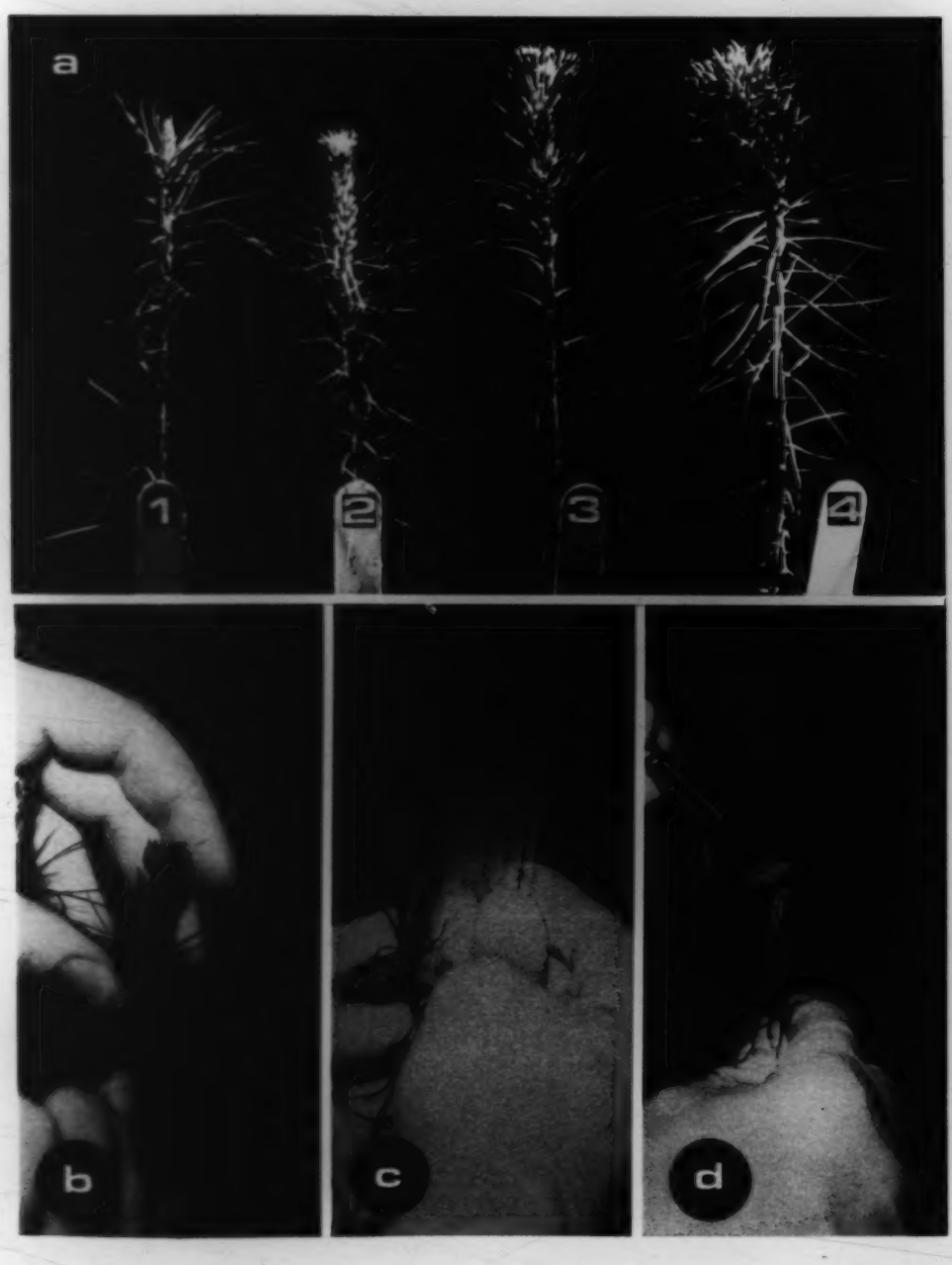
Figure 2.2. Greenhouse grown *Pinus contorta* seedlings at different stages of development used in controlled inoculations.

a. 1 = seedling 3 days after removal from cold storage, 2 = seedling 10 days post storage, 3 = seedling 17 days post storage, and 4 = seedling 24 days post storage.

b. Marking of seedling with non-toxic tree paint.

c. Assessing initial shoot length.

d. Inoculation of developing shoot with *E. harknessii* by brush method.


a
1
2
3
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b
c
d

length. Following inoculation, the trays were placed in wire mesh incubation cages, covered with moistened paper towels, sealed in separate opaque plastic bags, and incubated for 24 h at 18°C (Blenis and Pinnell 1991).

Immediately following incubation, seedlings were grouped into sets of three trees, called triplets, such that the elongating shoots within a triplet were similar in length and appearance. From one randomly selected tree per triplet, the expanding shoot was excised, while the remaining two trees were planted in separate 15-cm pots and returned to the greenhouse. After 5 months, seedlings in the greenhouse were evaluated for terminal shoot length, and gall formation and location (Blenis and Pinnell 1991). Percent elongation of the excised shoots was assumed to equal the average percent elongation of the other two trees in the triplet at the time of inoculation.

Data from all replicates over both years were combined and ten shoot elongation classes, each with approximately the same number of trees, were established. Relative susceptibility to infection in each class was defined as the number of galls in that class expressed as a percentage of the total number of galls. A non-linear spline or piece-wise regression (SAS Institute 1987) was used to model the relationship between relative susceptibility and shoot elongation. The plot of relative susceptibility vs. shoot elongation was then superimposed on the plots of spore production and spore viability vs. shoot elongation to determine which factor(s) were most important in limiting infection. Simple linear regression was used to determine if there was a relationship between gall location (defined as distance from the base of the shoot as a percentage of total shoot length) and shoot elongation at the time of inoculation. A non-linear spline was also used to determine the relationship between the number of galls on infected trees and shoot elongation class at the time of inoculation.

2.2.3 *Histology*

Histological studies were done to determine if the internal development of elongating shoots of greenhouse-grown seedlings was similar to the internal development of elongating

shoots in the field at the same stage of elongation. In 1992 and 1993, at both field sites, shoots were excised at weekly intervals over the growing season. They were taken from the same trees that were repeatedly measured to determine the relationship between date and shoot elongation. Shoot elongation of the field-collected shoots at the time of removal was assumed to be equal to the average shoot elongation of the repeatedly measured shoots at that time.

To assess development along the shoot length, 3 X 3 X 3 mm blocks were cut from the top, middle, and bottom of the preserved shoots. After fixing in FAA (formalin-acetic-acid), samples were rinsed in 50% ethanol, dehydrated in a graded *t*-butanol series under vacuum, and embedded in Paraplast-XTRA (Monoject Scientific, St. Louis, MO) under vacuum at 57°C. Tangential and cross sections of the samples were made on a rotary microtome, and mounted sequentially on glass slides using Haupt's adhesive. Paraffin was removed with xylene and sections were stained in Safranin-Fast Green (Jensen 1962). Slides were then examined under bright-field and phase-contrast microscopy.

2.3 RESULTS

2.3.1 *Spore production, spore viability, and shoot elongation in the field*

In 1992, spore production at site 1 was maximum when sampling began on June 1, by which time shoots were 62-70% elongated (Fig. 2.3a). At site 2, maximum sporulation occurred in the second week of June when shoots were 82-88% of their final lengths. At both sites, inoculum production declined as shoots elongated from 85 to 100% of their final lengths. By the first two weeks of July, few spores were caught and sampling ceased. Similar results were obtained in 1993, with sporulation gradually increasing during the second, third, and fourth weeks of May in both stands as shoots elongated to 45-60% of their final lengths. Peak sporulation occurred during the second week of June, when shoots reached 75-80% of their final lengths. As in 1992, spore levels in 1993 declined after the second week of June and fell to minimal levels by the first two weeks of July (Fig. 2.3b). During this time, shoots elongated from 80 to 100% of their final lengths.

Assessment of spore viability began once sufficient spores could be gathered from galls and continued for as long as spores were produced. This was done weekly from 20 May to 29 June 1992 and from 10 May to 1 July 1993. Spore viability remained above 80% in both stands in 1992 as shoots elongated from 70 to 100% of their final lengths (Fig. 2.3c). Spore viability remained above 91% in both stands in 1993 as shoot elongation increased from 55 to 100% (Fig. 2.3d).

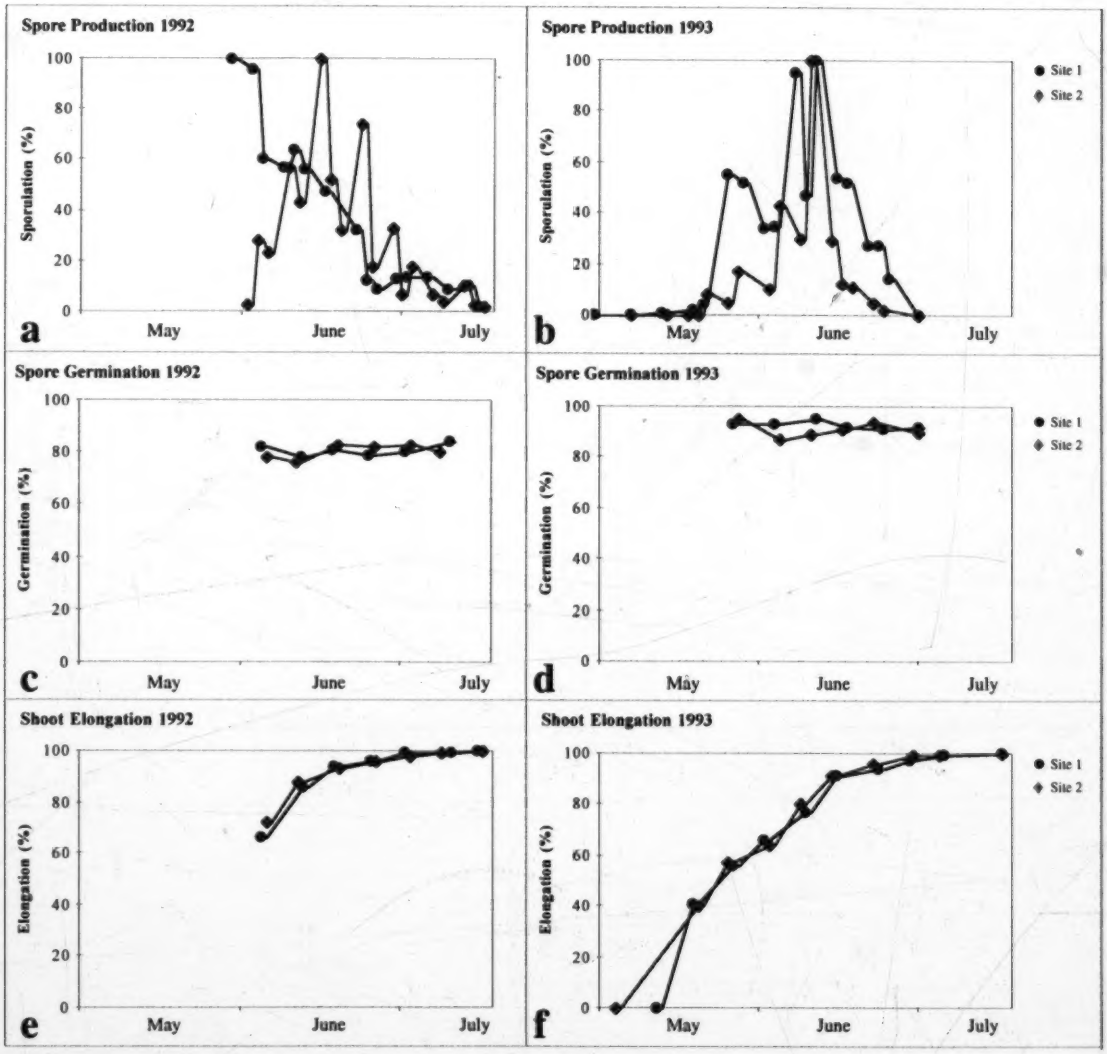
In both stands, and in both years, shoot elongation reached 90, 95, and 100% of final length by the third and fourth weeks of June, and the first week of July, respectively (Fig. 2.3 e & f). Coefficients of determination for the second order polynomials fitted through the shoot elongation data had R^2 values of 0.95 to 0.99 depending on year and site (Appendix 1).

2.3.2 *Greenhouse inoculations*

Germinabilility of combined spore lots used for the inoculations exceeded 80%. On average, 31% of the inoculated trees developed galls. Seedlings inoculated 24 days after removal from cold storage had < 10% infection, typically with a single gall developing near the distal end of infected shoots (Fig. 2.4a). Single, and to a lesser extent multiple galls, were observed on seedlings inoculated 17 days post-storage. When single infections occurred, they were often located higher on the shoot relative to galls on seedlings inoculated < 10 days post-storage. Multiple galls were frequently observed over the entire shoot of seedlings inoculated 3 or 10 days post-storage (Fig. 2.4b). There was little variation in final shoot length between the two trees per triplet for trees assigned to triplets 17 or 24 days post-storage (Fig. 2.4c). In contrast, grouping trees into triplets 3 or 10 days post-storage resulted in greater variability in final shoot length between the two trees.

To determine the relationship between stem elongation and infection frequency, data from the eight replicate inoculations were combined and 10 classes of shoot elongation, each with data for 49-50 trees per class, were created. Average stem elongation and percent infection were calculated for the ten classes, and a non-linear spline was used to predict percentage seedling infection as a function of stem elongation as follows:

Figure 2.3. Site 1 and site 2 field results for 1992 and 1993.



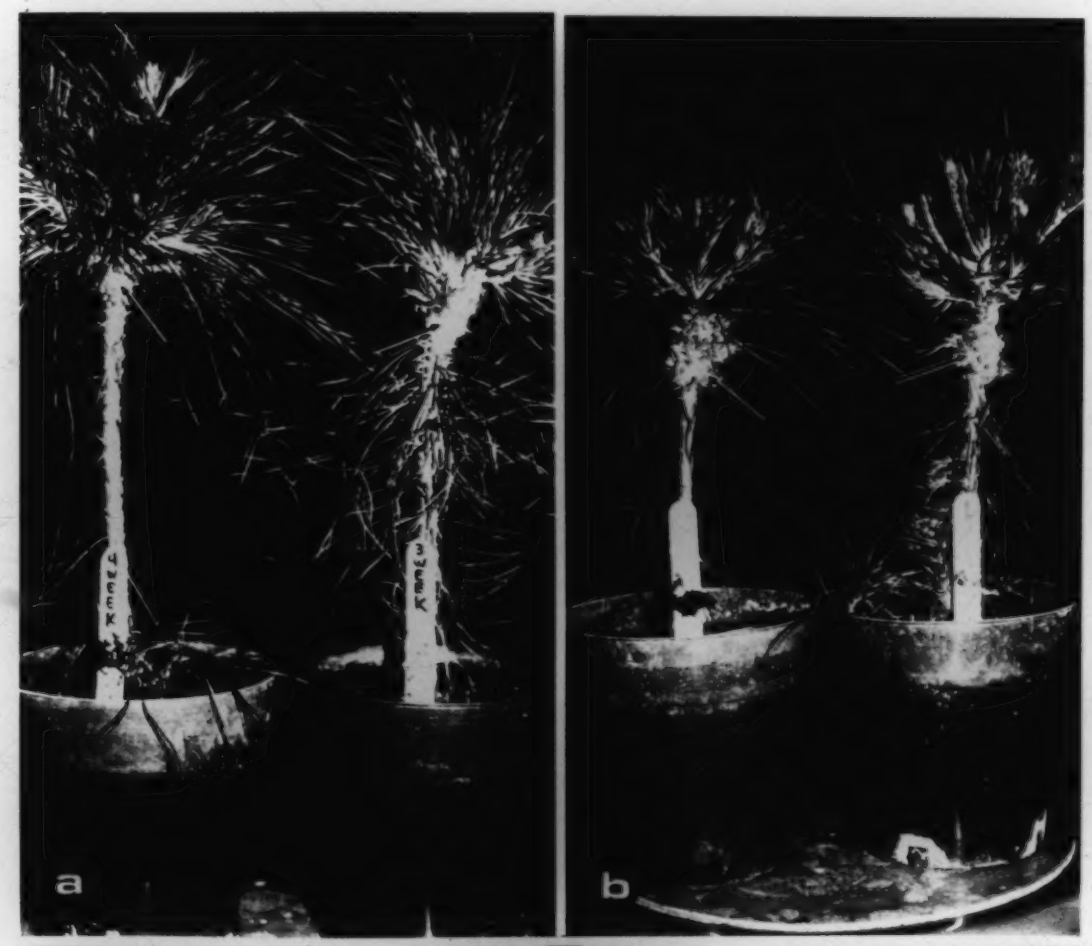
Spore production for Site 1 and Site 2 in 1992 (a) and 1993 (b). Spore germination for both sites in 1992 (c) and 1993 (d). Shoot elongation for both sites in 1992 (e) and 1993 (f).

Figure 2.4. Gall formation on seedling pairs following greenhouse inoculation in 1994-95. The third seedling of each triplet was used for histological examination.

a. Seedlings were 80% elongated at the time of inoculation. Arrows indicate gall location.

b. Seedlings were 40% elongated at the time of inoculation. Arrows indicate gall location. Note multiple gall formation on both seedlings.

c. Seedlings were 80% elongated at the time of inoculation. Note similar final lengths after 5 months.


a
b
c

$$Y = .70 + .10(X_1) - 6.62(X_2); K = .90$$

Where:

Y = predicted percent infection

K = shoot elongation at which predicted infection was maximum

X_1 = shoot elongation if shoot elongation ≤ K; 0 otherwise

X_2 = (shoot elongation - K) if shoot elongation ≥ K; 0 otherwise.

Thus, disease incidence gradually increased (slope = .10) as average shoot elongation increased from 31 to 90%. Percent infection rapidly dropped (slope = -6.52) to less than 10% as average shoot elongation increased from 90 to 100% (Fig. 2.5).

Gall location was linearly related to the degree of stem elongation at the time of inoculation (Fig. 2.6a). For example, on average, galls were formed 18%, 38%, and 70% of the way up the stem on seedlings that were 20%, 50% and fully elongated, respectively, at the time of inoculation. A non-linear spline was used to predict the average number of galls per infected tree as a function of average shoot elongation at the time of inoculation (Fig. 2.6b). Ten elongation classes, each with approximately 15 seedlings, were delineated. The final model for predicting gall frequency was,

$$Y = .28 + 7.65\ (X_1) - 14.22\ (X_2); K = .54$$

Where:

Y = gall frequency per infected tree

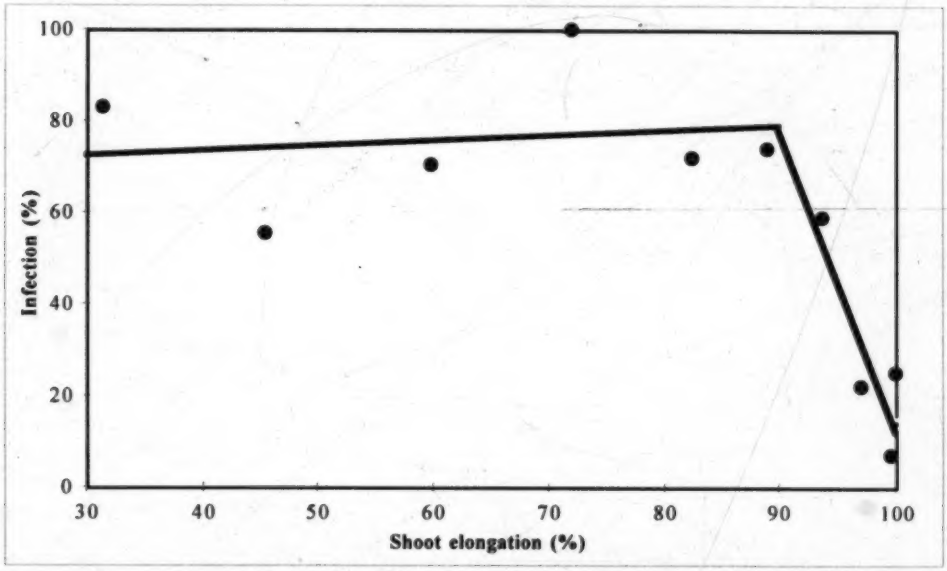
K = shoot elongation at which gall frequency per infected tree was maximum

X_1 = shoot elongation if shoot elongation ≤ K; 0 otherwise

X_2 = (shoot elongation - K) if shoot elongation ≥ K; 0 otherwise.

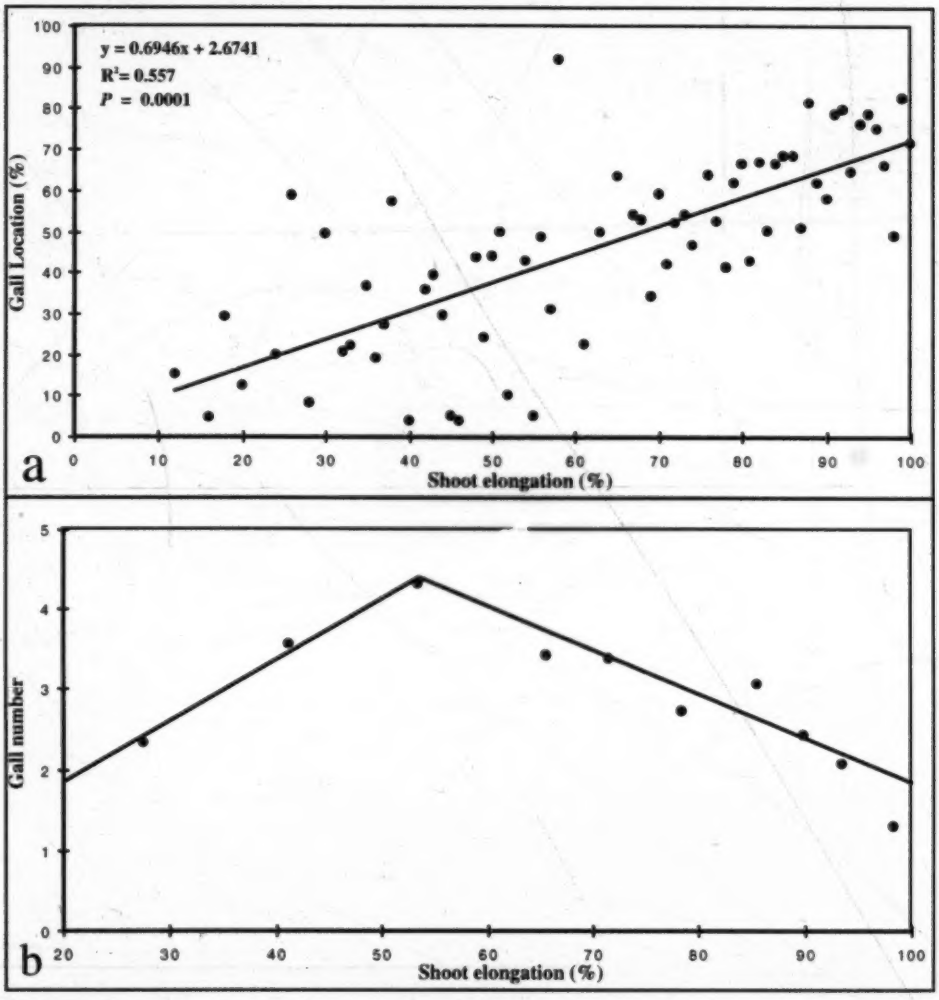
The terminals of infected trees in the first elongation class had grown to an average of 28% of their final length at the time of inoculation, and sustained an average of 2.3 galls. Multiple infections increased (slope = 7.65) as shoots expanded from 27 to 54% of their final lengths. The highest number of multiple infections (an average of 4.3 galls per tree) was associated with seedlings that were 54% elongated at the time of inoculation. A marked

Figure 2.5. Susceptibility to infection as a function of shoot elongation.



Percent infection was plotted against average shoot elongation for ten shoot elongation classes with 49-50 trees in each. The trendline represents predicted infection derived from a non-linear spline (SAS Institute 1987).

Figure 2.6. Regression of gall location and frequency vs shoot elongation.



Shoot elongation was defined as shoot length at inoculation as a percentage of final shoot length. a.) Gall location values of 0 and 100 percent represent the bottom and top of the shoots, respectively. Each point represents the average location of all galls on shoots with a given degree of elongation. The estimated standard deviation around each point was 22%. b.) The average number of galls per infected tree was plotted against average shoot elongation for ten shoot elongation classes. The trendline represents the predicted frequency derived from a non-linear spline.

decline (slope = -6.57) in multiple infections occurred thereafter as seedlings increased from 54 to 100% of their final lengths. All uninoculated seedlings had no galls, and growth of uninoculated and uninfected, inoculated trees was similar.

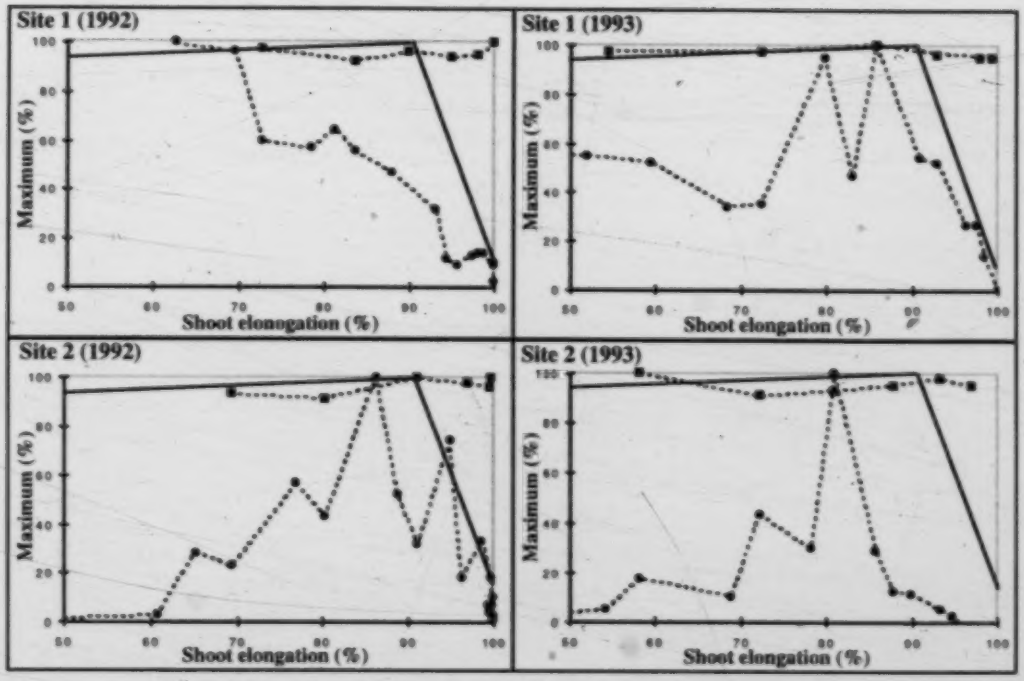
Simultaneous plotting of spore production, spore viability, and susceptibility data indicated that spore viability remained relatively constant over the growing season (Fig. 2.7). In contrast, spore production lagged behind relative susceptibility at the beginning of the growing season. At the end of the growing season, there was a marked decline in spore production that either preceded (site 1, 1992; site 2, 1993) or was approximately concurrent with (site 2, 1992; site 1, 1993) the decline in susceptibility (Fig. 2.7).

2.3.3 *Histology*

Percentage elongation of each greenhouse grown tree that was used for elongation was assumed to equal the average percentage elongation of the other two trees in the triplet, which were allowed to reach their full length. Percentage elongation of the field collected shoots was assumed to equal the average percent elongation of five repeatedly measured shoots from the same tree. Tissue differentiation increased from the bottom to the top of expanding shoots in both greenhouse grown and field collected samples. For example, there was no periderm in samples taken from the tops of shoots that were 80% elongated, whereas the bottom of the shoots had phellogen, phelloderm, and several layers of phellem. Therefore, histological comparisons between greenhouse and field samples were made from the middle of the shoots.

Patterns of vascular cambium differentiation in the middle of elongating shoots were similar for greenhouse grown and field grown shoots at the same stage of development (Fig. 2.8a - h). Shoots that were 30 to 40% elongated had vascular bundles and procambium, but no apparent vascular cambium (Fig. 2.8a & e). As differentiation progressed to 80%, there was an increase in secondary xylem in the vascular bundles and in most cases a conspicuous vascular cambium was apparent (Fig. 2.8b & f). By the time seedling elongation had reached 90%, a well established vascular cambium could be seen as the vascular bundles were replaced

Figure 2.7. Combined field and greenhouse results.



Susceptibility to infection as determined from greenhouse inoculations (—). Spore production for Site 1 and Site 2, 1992-1993 (●). Spore viability for Site 1 and Site 2, 1992-93 (■). All values are expressed as a percentage of the maximum predicted (susceptibility) or maximum observed (spore production and viability) values.

by additional secondary tissues (Fig. 2.8c & g). Fully elongated shoots had well developed vascular cambium and abundant secondary xylem (Fig. 2.8d & h).

Patterns of periderm formation, midway along the expanding shoot, were similar for greenhouse and field grown trees (Fig. 2.9a - h). The cuticle and epidermis were distinct, but no periderm had yet formed in shoots that had elongated to 30-40% their final lengths (Fig. 2.9a & e). Phellem, phellogen, and phelloderm became apparent once the shoots had reached 80% their final lengths (Fig. 2.9b & f). At 90% elongation, the cuticle and epidermis were suberized, phellogen and phelloderm were more conspicuous, and a thicker layer of phellem had formed (Fig. 2.8c & g). Fully elongated shoots were characterized by a well established periderm, more opaque cuticle, thicker suberized epidermis, phellogen, phelloderm, and extensive layers of phellem (Fig. 2.8d & h).

2.4 DISCUSSION

The first objective of this investigation was to determine the relative importance of spore production, spore viability, and shoot development in limiting rust infection. Spore viability was consistently high throughout the sampling period. It is important to note, however, that spores were sampled from galls that appeared to be free of hyperparasites. There are several species of fungi that parasitize *E. harknessii* galls (Byler *et al.* 1972; Tsuneda and Hiratsuka 1980) and the incidence of at least one of these, *S. uredinicola*, varies considerably among stands (Chapter 3). Thus, although spore viability will not likely be limiting where hyperparasitism is uncommon, reduced germinability will likely reduce infection in stands where hyperparasites are prevalent. In contrast to germinability, spore production initially lagged behind host susceptibility and later decreased prior to, or simultaneously with, the decrease in host susceptibility. At the beginning of June 1992 in site 2 and in late May at both sites, there were few spores present when shoots were 50-60% elongated (Fig. 2.3) which was the stage of maximum susceptibility (Fig. 2.4, 2.7). Spore production typically peaked in early to mid June at which time shoots were still very susceptible. Thereafter, as shoots reached 90% elongation, the combination of reduced

Figure. 2.8. Vascular cambium differentiation midway along expanding field collected and greenhouse grown shoots at similar stages of elongation (" vb = vascular bundles, pc = procambium, p = periderm, vc = vascular cambium).

a & e. Shoots 30% elongated, from field (a) and greenhouse (e). Note" initial vascular bundles, procambium, and the lack of vascular cambium. Bar = 500 μm.

b & f. Shoots 80% elongated, from field (b) and greenhouse (f) showing early development of vascular cambium and periderm. Bar = 500 μm.

c & g. Shoots 90% elongated, from field (c) and greenhouse (g) showing vascular cambium and periderm. Bar = 500 μm.

d & h. Shoots fully elongated, from field (d) and greenhouse (h) showing complete vascular cambium and a distinct periderm. Bar = 500 μm.

a
h
c
g
d
f
e

Figure 2.9. Periderm differentiation midway along expanding field collected and greenhouse grown shoots at similar stages of elongation ("c = cuticle, e = epidermis, ph = phellem, pl= phellogen, pd = phelloderm).

a & e. Shoots 30% elongated, from field (a) and greenhouse (e). Note" the cuticle and epidermis and the lack of periderm. Bar = 25 μm.

b & f. Shoots 80% elongated, from field (b) and greenhouse (f) showing the cuticle, epidermis, and initial periderm including phellem, phellogen, and phelloderm. Bar = 25 μm.

c & g. Shoots 90% elongated, from field (c) and greenhouse (g) showing cuticle, epidermis, and fully developed periderm including phellem, phellogen, and phelloderm. Bar = 25 μm.

d & h. Shoots fully elongated, from field (d) and greenhouse (h) showing cuticle, epidermis, and a thick layer of periderm consisting of phellem, phellogen, and phelloderm. Bar = 25 μm.

v
pd
pl
a
b
c
d
e
f
g
h

susceptibility and low spore production (Fig. 2.4, 2.7) would likely act together to greatly reduce infection. These findings are consistent with the report that susceptibility differed between two stages of shoot development (Kojwang and van der Kamp 1992), but provide greater detail on the association between infection and shoot elongation.

It is apparent that *P. contorta* undergoes a systematic change after breaking dormancy. Shoot elongation in the field was usually completed after the first 6 to 8 weeks following bud break. This is consistent with known sigmoidal growth patterns for other conifers (Evans and Biesemeyer 1988; Kozlowski and Ward 1961). Maturation of tissues begins first at the base, and progresses upward toward the distal region of the shoot (Cannell 1976; Owens and Molder 1984; Thompson 1976). This 'wave-like' pattern of development was associated with the location of galls on the shoot. Seedlings inoculated 24 days after removal from cold storage produced fewer galls which were located higher on the shoot than seedlings inoculated 3, 10, or 17 days post storage, respectively (Fig. 2.6a). In addition, greater numbers of multiple infections were commonly observed on seedlings inoculated 3 to 10 days post-storage (Fig. 2.6b). Therefore, it is possible that periderm maturing from the bottom upward on the shoot limits infection in a similar manner during the growing season.

The importance of periderm as a protective barrier to adverse effects from the environment, insects, and fungal pathogens has been well documented in conifers (Lewinsohn *et al.* 1991; Mullick and Jensen 1973; Struckmeyer and Riker 1951; True 1938). There is good evidence to suggest that periderm plays a significant role in resistance to pine stem rusts (Allen *et al.* 1990; True 1938; Miller *et al.* 1976; Walkinshsaw 1978). The fact that susceptibility is high prior to periderm formation supports the hypothesis that this is the particular developmental change that limits infection (True 1938). The continuous upward change in gall location for a given degree of shoot elongation (Fig. 2.6a) also illustrates a strong association between infection and the timing of periderm formation as shoots mature.

Evaluation of the relative importance of spore production, spore viability, and susceptibility (Fig. 2.7) rested upon the assumption that the relationship between susceptibil-

ity and shoot elongation under field conditions could be estimated using greenhouse grown trees. This assumption seems justified based on the histological studies which showed similar anatomical features for greenhouse grown and field grown trees at the same stage of shoot elongation (Fig 2.8, 2.9). However, these histological studies were in turn based on a second assumption, namely that the percent elongation of the excised shoot in each triplet was equal to the percent elongation of the remaining two shoots in the triplet. This assumption is somewhat more questionable given there was often considerable variation in final shoot length, and hence percent shoot elongation at the time of inoculation, between the two surviving trees per triplet. This variation was greatest for trees inoculated 3 to 10 days post-storage, perhaps due to differences in the size of shoot apices (Cannell 1976). Nevertheless, since shoots were approaching maximum susceptibility at this stage of development, violation of the assumption of equal elongation would have little effect on the conclusions. In contrast, for seedlings inoculated at later stages of development, when susceptibility was declining, there was much less variability between the remaining two triplets, thereby validating our conclusions.

Piece-wise regressions, sometimes referred to as splines or grafted polynomials, have been used as statistical tools in hypothesis testing (Gallant and Fuller 1973; Hotelling 1940; Smith 1979). This approach is sometimes criticized because an abrupt change in trend between two segments typically does not reflect the true change in a naturally occurring system. It is possible that the estimated points of maximum susceptibility and maximum number of multiple infections could be an overestimate or under estimate, respectively. However, the method seems acceptable given the reasonable expectation that infection frequency would initially increase with an increase in target area of the shoot and then decrease with periderm formation.

Environmental variation did not appear to dramatically influence spore production or viability in either site or year, nor did it change resulting shoot elongation, though one would expect greater differences to emerge if these variables were monitored over a period of many

years. Not all trees, nor all shoots on individual trees, elongate at the same rate (Kozlowski and Ward 1961). This may explain the variable infection rates between stands of lodgepole pine. Van der Kamp and Tait (1990) suggested the pathosystem is in a state of dynamic equilibrium and that low disease incidence may be attributable to other non-genetic factors such as poor spore dispersal and variable microclimates. We have demonstrated that despite the variation in microclimate, *P. contorta* development, and *E. harknessii* aggressiveness, it is possible to construct a model for disease progression in the greenhouse and correlate the findings to spore production, spore viability, and shoot development in the field (Fig. 2.7).

These findings indicate that chemical control of the disease may be more effective if treatment occurs after spore release and prior to shoots reaching 90% of their final development. Moreover, only mycoparasites operating prior to shoots growing to 90% will have a direct negative impact on the level of rust infection. *Scytalidium uredinicola* has been shown to be active as early as April in west-central Alberta (Chapter 4), and in fact may contribute to the decline in spore production as the growing season progresses (Kuhlman 1981).

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CHAPTER 3

EFFECTS OF GALL AGE, GALL SIZE, AND RUST SEVERITY ON INCIDENCE OF THE MYCOPARASITE *SCYTALIDIUM UREDINICOLA*

3.1 INTRODUCTION

Numerous fungi, insects, slugs, and rodents have been identified as natural control agents of pine stem rusts (Bergdahl and French 1978; Byler *et al.* 1972*a*; 1972*b*; Hedgcock and Hunt 1920; Hiratsuka *et al.* 1979; Hunt 1978; Kuhlman 1981*a* and 1981*b*; Parsons 1967; Powell 1971*a*; 1971*b*; Salt and Roth 1980; Wong 1972). The potential use of such agents for biological control has been discussed and may offer an alternative to chemical, silvicultural, and genetic controls for managing this group of pine stem diseases (Ayer *et al.* 1980; Byler and Cobb 1969; Hiratsuka 1991; Kuhlman *et al.* 1976; Pickard *et al.* 1983; Wicker 1981). Several gall-inhabiting fungi in particular are known to parasitize *Endocronartium harknessii* (J. P. Moore) Y. Hiratsuka, the cause of western gall rust, and are suspected to be involved in the deactivation of this rust over time (Byler *et al.* 1972*a*; 1972*b*; Currie and Hiratsuka 1996; Hiratsuka 1979; Hiratsuka *et al.* 1987; Sutton 1973; Tsuneda and Hiratsuka 1981*a*; Tsuneda and Hiratsuka 1982). However, most evidence for biological control of western gall rust comes from laboratory experiments, and little is known about factors influencing hyperparasites in the field (Hiratsuka and Powell 1976; Tsuneda and Hiratsuka 1981*b*).

Scytalidium uredinicola Kuhlman *et al.* is considered the most destructive hyperparasite of *E. harknessii* in western Canada (Hiratsuka 1979; Hiratsuka *et al.* 1987; Tsuneda and Hiratsuka 1980; Tsuneda *et al.* 1980). This arthroconidial fungus is vectored by the beetle *Epuraea obliquus* Hatch (Coleoptera: Nitidulidae), which inhabits galls formed by *E. harknessii* (Chakravarty and Hiratsuka 1995; Currie 1995; Currie and Hiratsuka 1996; Currie *et al.* 1995). Once established in a gall, *S. uredinicola* is presumed to overwinter on the outer bark and beneath the last formed periderm produced in the fall (Moltzan *et al.* 1995; Tsuneda and Hiratsuka 1981*b*). The hyperparasite is active early in the spring at the onset of western gall rust sporulation, when trees are most susceptible to the rust. For these reasons, *S. uredinicola* may be important in regulating the gall rust pathosystem (van der Kamp and Blenis 1996), and may be the best candidate for the biological control of western gall rust

(Hiratsuka 1991).

Hyperparasites have been modeled as density-dependent constraints of western gall rust (van der Kamp and Blenis 1996). Before the importance of hyperparasites in maintaining gall rust incidence about some equilibrium can be evaluated, the epidemiology of individual organisms like *S. uredinicola*, and factors that influence its occurrence must be determined. In west-central Alberta, 75-80% of galls surveyed were reported to have *S. uredinicola* (Currie and Hiratsuka 1996; Tsuneda *et al.* 1980). However, it remains uncertain whether such high levels of hyperparasitism are typical for this region. Hence, the first objective of this study was to determine the incidence of *S. uredinicola* in west-central Alberta.

The behavior of a multilevel pathosystem (host-pathogen-insect-mycoparasite) will undoubtedly be complex and perhaps difficult to define. Information on the relationship between such factors as gall age or size and hyperparasite incidence may be useful for testing assumptions and improving models of gall rust population dynamics. Furthermore, if a strong correlation between *S. uredinicola* and these factors could be established, then the ability to specifically target this naturally occurring control would be improved. Finally, since western gall rust levels vary from site to site, it is necessary to determine what impact, if any, gall rust incidence has on the level of *S. uredinicola* in stands. Therefore, a second objective was to test the hypothesis that gall age, gall size, and rust severity are associated with the incidence of *S. uredinicola*.

3.2 MATERIALS AND METHODS

3.2.1 *Field survey and gall collection*

Sixteen *Pinus contorta* Dougl. ex Loud. stands were surveyed for western gall rust between 1991 to 1993 (Blenis and Duncan 1997). The stands were located in west-central Alberta and sampling was distributed over 4800 ha and these were spread across three administrative areas known as working circles, within the Weldwood of Canada Forest Management Area (FMA). Stands were 11-86 ha in size, and 19-32 years in age when

surveyed. Main stem galls were counted and aged on five trees in each of 10 equally spaced plots along a transect through the center of each stand. In 1995 and 1996, a survey for *S. uredinicola* was conducted in these 16 stands, plus an additional two stands that had not been surveyed for western gall rust. All stands had experienced a single season of heavy infection or 'wave year' (Peterson 1971) of infection between the ages of 19-22 years (Table 1 in Blenis and Duncan 1997). A single gall was randomly selected, without regard to whether it was a branch or stem gall, from 20 trees that were 60 m apart along a single line transect in each stand. The galls were placed in cold storage (-20°C) until needed. Gall age was determined by counting the annual growth rings with a dissecting microscope and gall size was measured using a string wrapped around the middle circumference of the gall.

3.2.2 ***Fungal determination***

To determine the presence of *S. uredinicola* in galls, layers of exfoliating aecia-bearing tissues (True 1938) were placed on carrot agar media. If galls were < 5 cm in diameter, 1-2 g of exfoliating aecia-bearing tissue was randomly selected from the entire gall surface. If gall diameter was > 5 cm, 0.3-0.7 g of exfoliated tissue was taken from each of three randomly selected areas on the gall. The gall tissue was placed in a sterile porcelain mortar, flooded with liquid nitrogen (-180°C), and crushed to a fine powder with a pestle. Internal gall tissues, consisting of the periderm, secondary phloem, vascular cambium, and secondary xylem, were similarly sampled, then surface sterilized in a 1:1 mixture of bleach / sterile distilled water for 30 s before being pulverized as above. The crushed material was transferred to 60 X 15 mm petri plates, shaken to ensure even mixture, uniformly distributed over carrot agar medium in 100 X 15 mm petri plates, incubated at 18°C in the dark, and evaluated after 3-4 days. Putative *S. uredinicola* colonies were subcultured and grown for 2 weeks as above. Identification of *S. uredinicola* was confirmed by comparison using a light microscope with known isolates obtained from the Canadian Forestry Service, Northern Foresty Centre, Edmonton, Alberta Canada (NOF #1885 and NOF #1868).

3.2.3 *Data analysis*

A gall was considered to have *S. uredinicola* if the hyperparasite was recovered from either external or internal gall tissue. The effect of gall age and gall size, with stand as a blocking factor, on the presence of *S. uredinicola* was analyzed using logistic regression (SAS Institute 1989; Hosmer and Lemeshow 1989). Partial plots were used to depict the association between 1) *S. uredinicola* and gall age, adjusted for gall size and 2) between *S. uredinicola* infection and gall size, adjusted for gall age (Jobson 1991).

Rust severity for each stand was expressed as area under the disease progress curve AUDPC (Shaner and Finney 1977) given,

$$\text{AUDPC} = r_1 / 2 + \sum_{i=2}^{n} [(r_{i-1} + r_i) / 2]$$

Where:

r_i = rust severity (stem galls / tree) at stand age i

n = stand age at survey.

Regression analysis was used to model the proportion of galls in each stand with *S. uredinicola* as a function of AUDPC. Average gall age and average gall size were included as predictors in the model so that the association between *S. uredinicola* and rust severity would not be confounded by those two factors.

3.3 RESULTS

The 360 galls from 18 stands ranged in age from 3 to 16 years (CV = 32%) and in diameter from 0.8 to 9.7 cm (CV = 48%). On average, 39% of the galls yielded *S. uredinicola* (Table 3.1), with the rate of recovery being greater from external tissues (75%) than internal tissues (16%).

The final logistic model for predicting *S. uredinicola* was,

$$C = -9.50 + \sum y_i \text{ (stand}_i\text{)} + 0.28(\text{age}) + 0.77(\text{size})$$

Where:

C = ln(odds of *S. uredinicola* being present on a gall)

y_i = the effect of the ith stand on C

Table 3.1. Proportion of galls parasitized by *Scytalidium uredinicola*, average gall age, average gall diameter, and AUDPC for 18 stands in three working circles.

Stand	*S. uredinicola*	Gall age (yrs)	Gall size (cm)	AUDPC
Working Circle I				
1	0.20	7.5	3.26	31.4
2	0.45	9.9	4.74	21.4
3	0.45	9.7	4.66	20.7
4	0.15	7.7	2.70	41.1
5	0.55	7.6	2.75	37.7
6	0.40	6.4	3.07	- *
Working Circle II				
1	0.60	7.7	3.39	175.3
2	0.30	9.3	3.28	20.3
3	0.15	6.8	2.87	13.5
4	0.60	10.9	4.75	14.3
5	0.50	8.9	3.57	21.0
6	0.65	10.2	4.30	25.1
7	0.50	10.3	4.45	26.7
8	0.40	10.8	4.44	27.8
9	0.35	7.5	3.40	-
Working Circle III				
1	0.25	7.6	3.83	5.1
2	0.45	10.4	4.13	5.3
3	0.10	11.5	3.38	9.3

* Stand not surveyed for gall rust.

$stand_i$ = an indicator variable that equals 1 for the *i*th site and 0 otherwise with the sixteenth stand arbitrarily set to 0 and used as a reference site

age = gall age in years

size = gall diameter in centimeters.

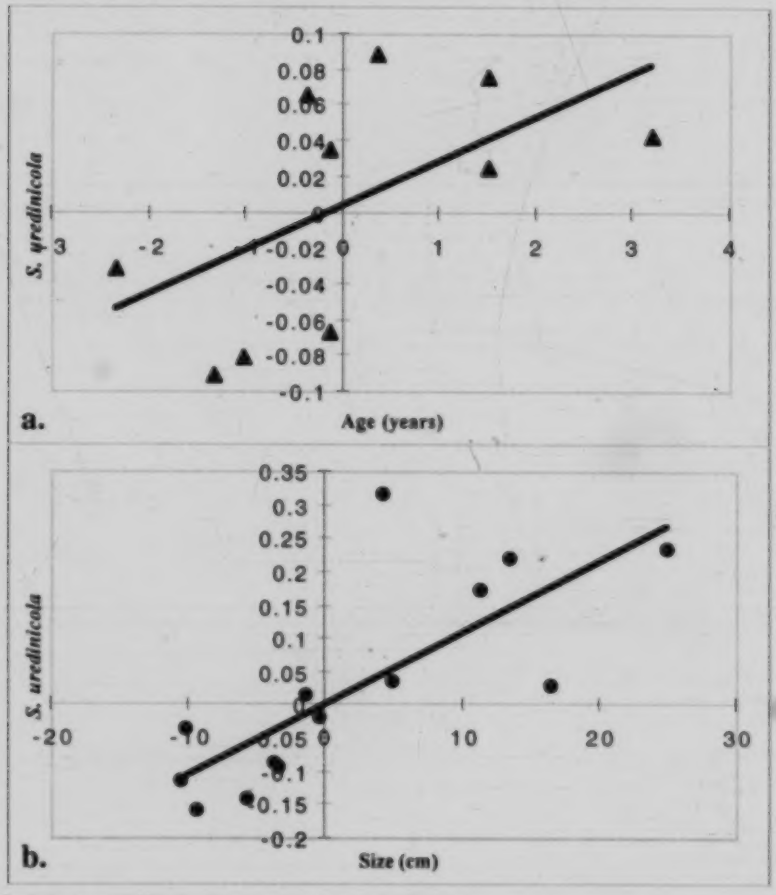
The calculated goodness-of-fit statistic was 5.70, corresponding to a *P* value of 0.68 indicating no significant difference between observed and expected values. Stand, gall age, and gall size were all significant at $P = 0.0001$, 0.0002, and 0.0001, respectively, denoting a positive association between hyperparasitism and gall age for a given gall size, and an association between hyperparasitism and gall size for a given gall age (Figure 3.1a, b). The regression coefficient for age of 0.28 indicates that for each one year increase in gall age, the odds of a gall being parasitized by *S. uredinicola* increases by a factor of $e^{0.28}$, or 1.3. The regression coefficient of 0.77 indicates for each 1 cm increase in gall diameter, the odds of hyperparasitism increased by a factor of $e^{0.77}$, or 2.1. In addition to *S. uredinicola*, several other fungi were isolated from galls including *Aureobasidium pullulans* (DeBary) Arnaud, *Cladosporium gallicola* Sutton, *Monocillium nordinii* (Bourchier) W. Gams., *Penicillium* sp., *Alternaria* sp., *Fusarium* sp., and others.

Values of AUDPC ranged between 5.1 and 175.3. The occurrence of *S. uredinicola* was only weakly associated with AUDPC of gall rust infection (Fig. 3.2). Stand 1 in working circle II had a considerably higher rust severity than any of the other stands, and therefore strongly influenced the regression equation. No association between *S. uredinicola* and rust severity could be detected if this stand was excluded from the analysis.

3.4 DISCUSSION

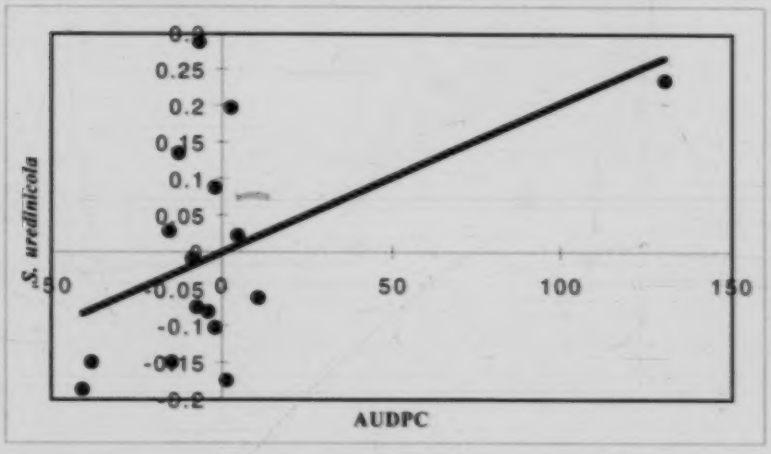
Scytalidium uredinicola was present in each surveyed stand, indicating that this hyperparasite is extensively distributed in west-central Alberta. However, the incidence among individual stands was quite variable (Table 3.1). On average, *S. uredinicola* was recovered from 39% of the galls. This is in contrast to reports of 75-80% infection in the same FMA (Currie and Hiratsuka 1996; Tsuneda *et al.* 1980). The reasons for the higher values in previous studies could be that other fungi such as *Penicillium uredineicolum* A.

Figure 3.1. Association between gall size and age and proportion of galls infected by *Scytalidium uredinicola*.



a.) Partial plot of the residuals of the proportion of galls infected by *S. uredinicola* and gall age, adjusted for gall size. b) Partial plot of the residuals of the proportion of galls infected by *S. uredinicola* and gall size, adjusted for gall age.

Figure 3.2. Association between rust severity and the proportion of galls infected by *Scytalidium uredinicola*.



Partial plot of the residuals of the proportion of galls infected by *S. uredinicola* and area under the disease progress curve (AUDPC), adjusted for gall age and gall size.

Hulea can appear light green in color in invaded aecia (Byler *et al.* 1972*a*), confounding the visual identification of *S. uredinicola* (Hiratsuka *et al.* 1979; Kuhlman *et al.* 1976), or galls may not have been randomly sampled. In this study, isolation rather than visual evaluation was used to assess the occurrence of *S. uredinicola*. Although this method precludes the problem of over-estimation through possible misidentification, it might have resulted in underestimation if other fungi on the gall proliferated faster on petri plates, obscuring the presence of *S. uredinicola*. It should be noted that *S. uredinicola* grew slowly on carrot agar, and was often overgrown by *A. pullulans*, *C. gallicola*, *Penicillium* sp., and other fungi, especially on cultures from external tissues. However, since it was always possible to isolate *S. uredinicola* from galls on which presence of the hyperparasite had been confirmed by microscopy, the risk of underestimation would appear to be low.

Since most surveyed stands were uniformly infected during 'wave years' (Blenis and Duncan 1997; Peterson 1971), only a narrow range of gall ages was sampled. Conversely, gall size within the 18 stands was more variable, because the sample included both stem galls, which can be quite large and branch galls, which typically are much smaller. Although gall age and size were positively correlated, the odds of hyperparasitism increased with age by a factor of 1.3 for each year of growth, even for galls of similar size. In addition, there was a partial correlation between gall size and infection, with the odds of recovering *S. uredinicola* increasing by a factor of 2.1 with each 1 cm increase in gall diameter.

There are several possible reasons why the incidence of *S. uredinicola* would increase with gall age. First, because *S. uredinicola* can overwinter beneath the periderm of infected galls (Moltzan *et al.* 1995), incidence would likely be cumulative. Secondly, *E. obliquus* was attracted to galls in field releases and laboratory tests (Chakravarty and Hiratsuka 1995; Currie and Hiratsuka 1996), possibly due to a chemical or color stimulus. More time enables more gall-associated fungi to become established on galls. This in turn might attract more insects to the gall and also provide a larger source of nutrition for developing larvae (Nelson

1982; Powell 1971*a*; Powell 1982; Wong 1972). Thirdly, developmental changes associated with gall aging also may increase infection frequency. As aecia become active in the spring they push up as more aeciospores form below, causing the host tissues bearing aecia from the previous year to scale off as sporulation begins. As sporulation ends, the formation of a new periderm is completed by fall and separation of aecia-bearing tissues begins, culminating before winter (True 1938) or prior to March (personal observation). It is possible that complete exfoliation of the aecia-bearing tissues on young (2- to 5-year old) galls prevents *S. uredinicola* from building up on the surface of the gall. Conversely, mature (> 6-year-old) galls form many subsequent periderms and retain most of their exfoliating tissues, allowing the mycoparasite to accumulate. In addition, the numerous cracks, fissures, and leftover periderms remaining on the gall surface provide cover for the vector *E. obliquus*. Finally, it is possible that a succession of microorganisms might be necessary before *S. uredinicola* can become established on the gall. Fungal interactions have been investigated for other plant surfaces (Andrews 1992; Baker 1987; Cook 1996) but are not well understood for galls. Results presented here and by others show that *A. pullulans* and *C. gallicola* are very frequent on the surface of the gall regardless of age or size (Tsuneda and Hiratsuka 1979; Tsuneda and Hiratsuka 1982). The extent to which *S. uredinicola* depends on other fungal colonizers, such at these, needs further investigation, because their succession may be important in establishing natural control of western gall rust.

Some of the factors which cause the incidence of *S. uredinicola* to increase with gall age may also be responsible for causing hyperparasitism to increase with gall size for equally aged galls. For example, it seems likely that larger sized galls would be more likely to attract the vector of *S. uredinicola*. In addition, for any given age, main stem galls tend to be larger than branch galls and hence some of the apparent effect of gall size may be a consequence of differences in where the galls formed.

AUDPC was only weakly associated with the occurrence of *S. uredinicola* (Fig. 3.2), and the association was only detectable if one particularly heavily infected stand was included

in the analysis. Nevertheless, the possibility that the hyperparasite is acting as a density dependent constraint of gall rust cannot be discounted. Even if *S. uredinicola* was acting as a density dependent constraint, there might not necessarily be a correlation between the incidence of the rust and its parasite. If stands were asynchronous with respect to stand age and wave year, the lag time between the occurrence of *E. harknessii* and *S. uredinicola* might prevent detection of a correlation. Nevertheless, stands in this survey (Table 3.1) were uniform in age and had experienced a wave year at about the same time in the life of the stand (Blenis and Duncan 1997). Thus, the weak statistical association may be indicative of a weak biological relationship.

In summary, it is apparent that older galls are seldom free from invasion by other fungi in west-central Alberta as reported by others (Currie and Hiratsuka 1996; Hiratsuka *et al.* 1987; Tsuneda and Hiratsuka 1979; Tsuneda and Hiratsuka 1981*b*; Tsuneda *et al.* 1980). However, the extremely high levels of *S. uredinicola* that previously have been reported (Currie and Hiratsuka 1996; Tsuneda *et al.* 1980) may not be occurring uniformly throughout this region. The frequency of *S. uredinicola* was associated with gall age and size, in spite of all other factors such as microclimate within the stand, occurrence of the insect vector, fungal interactions, etc. that would influence the likelihood of hyperparasitism. *Scytalidium uredinicola* has not been observed in heavily infected young stands even on large-diameter galls (personal observation) and this study confirms that the hyperparasite does not become established early in gall rust epidemics. This lag in establishment may be a significant obstacle to the use of *S. uredinicola* as a biological control agent. The weak association between gall rust severity and the incidence of *S. uredinicola* suggests that the occurrence of the hyperparasite does not increase with increasing levels of western gall rust. Nevertheless, it is still possible that the hyperparasite is acting as a density dependent constraint of the rust. Further efforts to resolve this issue are justifiable, since density dependent and density independent pathosystems may respond differently to perturbations.

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CHAPTER 4

SEASONAL DEVELOPMENT AND IMPACT OF *SCYTALIDIUM UREDINICOLA*, A MYCOPARASITE ON WESTERN GALL RUST

4.1 INTRODUCTION

Western gall rust caused by *Endocronartium harknessii* (J.P. Moore) Y. Hiratsuka is an important stem disease of hard pines in North America (Ziller 1974). Infections can take place either on lateral or main stem shoots. The impact of branch galls on tree growth is negligible, whereas stem infections can kill trees prior to stand rotation (Gross 1983; van der Kamp 1994). Control measures such as genetic resistance, fungicide treatment, and pruning have been suggested, but results of previous experiments often have been inconclusive or the methods economically impractical (Hunt 1982; Kistler and Merrill 1978; Leaphart and Wicker 1968; Merrill and Kistler 1976). In order to successfully cultivate hard pines, development of cost efficient and environmentally acceptable management strategies for western gall rust will be required.

There are a number of gall-inhabiting fungi that appear to limit the incidence of western gall rust (Bergdahl and French 1978; Byler *et al.* 1972*a* and 1972*b*; Hedgcock and Hunt 1920; Hiratsuka 1979; Hunt 1978; Kuhlman 1981*a* and 1981*b*; Parsons 1967; Powell 1971*a* and 1971*b*; Salt and Roth 1980; Wong 1972). Many of these fungi have been associated with cambial or rust mortality, and may have the potential to control the disease (Byler *et al.* 1972*a* and 1972*b*; Hiratsuka 1979; Hiratsuka *et al.* 1987; Sutton 1973; Tsuneda and Hiratsuka 1981*a*, and 1981*b*; Tsuneda and Hiratsuka 1982). A strategy recently has been proposed that would make use of mycoparasites vectored by certain free-moving, gall-seeking insects to control of western gall rust (Hiratsuka 1991; Currie and Hiratsuka 1996). However, before such a control system can be undertaken in the field, more information on the seasonal development of the candidate fungi is needed.

Scytalidium uredinicola Kuhlman *et al.* is a mycoparasite that is capable of reducing the viability of western gall rust spores under laboratory conditions (Tsuneda *et al.* 1980). In Canada, the fungus is vectored by *Epuraea obliquus* Hatch (Coleoptera: Nitidulidae), a gall-inhabiting beetle (Currie 1995; Currie and Hiratsuka 1996). *Scytalidium uredinicola* has been detected on sporulating galls by its gray-buffy green color amid the normally bright yellow-

orange aeciospores of *E. harknessii* (Hiratsuka 1979; Kuhlman *et al.* 1976). Parasitism is accompanied by diffusion of chemicals or enzymes in advance of the mycoparasite's growth (Chakravarty and Hiratsuka 1995; Tsuneda *et al.* 1980). Tsuneda and co-workers (1980) further demonstrated that hyphae of *S. uredinicola* appeared to penetrate the rust sorus to a depth of 300 μm into the gall, inactivating both mature and immature spore layers. This is in contrast to *Monocillium nordinii* (Bourchier) W. Gams. and *Cladosporium gallicola* Sutton, both of which are surface mycoparasites of *E. harknessii* (Hiratsuka 1979; Tsuneda and Hiratsuka 1981*a*).

Whereas the mode of parasitism of *S. uredinicola* has been thoroughly investigated, information on its location on or in the gall over the growing season is generally lacking. Because investigators have focused exclusively on sporulating rust sori, it remains unclear whether the hyperparasite is perennial, or whether it must be re-established annually on the gall. A better understanding of the parasitic activity of *S. uredinicola* may help clarify when and where the mycoparasite should be introduced if it is to be used as a biological control agent. For example, parasitism of sori would likely reduce infection of pines if the decrease in gall rust inoculum occurred when trees were susceptible. However, if parasitism occurred later in the sporulating season when the host was quite resistant, then the effect on the rust population might be negligible. For a given gall size, the odds of a gall being parasitized increased by a factor of 1.3 for each year increase in gall age (Chapter 3). Thus, if the location and development of the *S. uredinicola* throughout the year is to be studied, it may be useful to consider whether its occurrence is influenced by gall age.

Therefore, to more completely describe the life history of *S. uredinicola*, a study was done (i) to determine whether the occurrence of *S. uredinicola* in external and internal tissues throughout the growing season varied with gall age, (ii) to describe the location and development of *S. uredinicola* throughout the growing season on main stem galls, and (iii) to examine the effect of parasitism by *S. uredinicola* on the viability of gall rust spores in the field.

4.2 MATERIALS AND METHODS

4.2.1 *Sites*

Field studies were conducted between 1993-96 in two heavily rust-infected stands of lodgepole pine (*Pinus contorta* Doug. var. *latifolia* Engelm.) located 36 km northeast and 25 km southwest of Hinton, Alberta. Sites were 19- and 20-years-old, 21.3 and 86 ha in size, and thinned to approximately 2500 stems per hectare in 1985 and 1987, respectively.

4.2.2 *Occurrence and gall age*

Five galls in each of three age classes 3-5, 6-9, and 10+ years, were removed from trees along a 60 m straight-line transect in both stands. Collections were made once a month over an entire year to obtain a total sample of 360 galls. The galls were kept frozen (-20°C) until needed. To determine if *S. uredinicola* was present on the gall surface, layers of exfoliated aecia-bearing tissues (True 1938) were cultured on carrot agar media, as described in Chapter 3. To determine if *S. uredinicola* was present inside the gall, wood chips were cut from beneath the last formed periderm (Byler *et al.* 1971*a*), surface sterilized in alcohol, then placed on carrot agar plates that were incubated at 18°C in the dark. After 3 days, fungal colonies were subcultured and grown for 2 weeks. Wet mounts were prepared from subcultures, stained with lactophenol cotton blue, and identified with a microscope at a magnification of 400X. The Wilcoxon signed rank test was used to determine if there was a difference in parasitism between age classes and between internal and external tissues (SAS Institute 1989). In March, an additional five galls per age class were collected for histological examination.

4.2.3 *Seasonal development*

Three methods light microscopy, scanning electron microscopy, and field examination were used to investigate the seasonal development of *S. uredinicola*. For histology and SEM, 24 trees with main stem galls greater than 10 years old were located in each site during 1993. Parasitized areas on the galls were marked, and the presence of *S. uredinicola* was confirmed by using a sterile forceps to transfer a small mass of the

hyperparasite to carrot agar plates, which were incubated as described in Chapter 3. This was done during the last week of May and the beginning of June, when rust sporulation was apparent and infected sori could be detected (Fig. 4.1a). Parasitized tissues were destructively sampled from the galls using a #6 cork borer inserted to a depth of 1.5 cm. Two to three plugs were removed randomly each month from one or two different main stem infections per stand between 1994-1995. For comparison, one to three plugs were taken from 3- to 5-year-old galls that appeared to be non-parasitized and developing as normal gall rust infections (Fig. 4.1b). All excised plugs were preserved immediately in formalin acetic alcohol (FAA) for use in histology and scanning electron microscopy (Jensen 1962).

Small blocks (5 x 5 x 3 mm) of gall tissue were cut from the plugs for both histological and SEM evaluation. For histology, samples were rinsed in 50% ethanol and then placed in either 10% ethylenediamine (Carlquist 1982), or fabric softener (C. Walkinshaw, personal communication) for 1 week, dehydrated in a graded t-butanol series under vacuum and embedded in Paraplast-XTRA (Monoject Scientific, St. Louis, MO) under vacuum at 60°C. Tangential and cross sections of the samples were made on a rotary microtome, and mounted sequentially on glass slides using Haupt's adhesive. Paraffin was removed with xylene and sections were then stained with picro-aniline blue (Jensen 1962; Jewell 1958). Slides were examined under bright field microscopy. For scanning electron microscopy, blocks of tissue were fixed 12 h overnight with osmium tetroxide and then prior to examination mounted on metal stubs and sputter coated with approximately 200 angstroms of gold palladium and examined with a Cambridge Steroscan S_4 scanning electron microscope.

Field examinations of the 15 parasitized main stem galls, not destructively sampled as above, were made between late April and early May from 1993-1995, to determine the appearance and location of *S. uredinicola* as *E. harknessii* was beginning to sporulate.

4.2.4 *Rust spore viability*

The effect of *S. uredinicola* on the germinability of gall rust spores was evaluated for 20 additional main stem galls, 10 per site in each of 1993 and 1994. Samples were taken

Figure. 4.1. Comparison of *S. uredinicola*-parasitized vs non-parasitized aecia of *E. harknessii*.

a. A 10-year-old main stem infection of *E. harknessii* parasitized by *S. uredinicola*. Note the grey-green area (red pin) showing the mycoparasite's location and the bright yellow-orange area (blue pin) of normally sporulating western gall rust.

b. A 4-year-old main stem infection of *E. harknessii* with no apparent mycoparasitism.

b

a

from the same gall from 26 May to 5 July 1993, and from 9 June to 14 July 1994. From parasitized and non-parasitized sori on the same gall, aeciospores were removed from the sori with a sterile scalpel and dispersed with a short burst of air onto 1.5% water agar plates. Percent germination of 100 spores after 12 h was determined as described in Chapter 2.

4.3 RESULTS

4.3.1 *Occurrence and gall age*

Scytalidium uredinicola was not isolated from either external or internal samples taken from 3- to 5-year-old galls during the 12-month study (Table 4.1). *Cladosporium gallicola* was the most commonly recovered fungus for this age class, occurring more often on exfoliated tissues than internal tissues for all sampling dates. *Scytalidium uredinicola* was isolated frequently from the external surface, but never from internal samples, for 6- to 9-year-old galls. Other fungi such as *Aureobasidium pullulans* (DeBary) Arnaud, *C. gallicola*, *M. nordinii*, *Penicillium* sp., *Alternaria* sp., *Fusarium* sp., were also isolated, more often from external rather than internal tissues over all sampling dates. Samples taken from galls greater than 10 years of age had *S. uredinicola* present in both external and internal tissues. The hyperparasite was more common on the external tissues of this age class than on 6- to 9-year-old galls and more common on external than internal tissues (Table 4.1). In addition, all of the above mentioned other fungi were also isolated.

Three- to five-year-old galls collected in March had a smooth, continuous periderm with few cracks or fissures. Cross sections taken at the perimeter of the gall showed a thick layer of secondary phloem that was 4-5 μm thick. Areas of tissue that had previously sporulated did not accumulate on the gall and were completely exfoliated as a result of periderm formation. No evidence of *S. uredinicola* was detected in the secondary phloem from galls in this age class. Galls in the 6- to 9-year-old age class had more breaks and fissures in the periderm than younger galls, and secondary phloem was not as thick on the perimeter, measuring 2-3 μm. In this age class, 1-2 seasons of colonized aecia persisted on the gall surface. Evidence of fungal activity was visible on most of the exfoliating tissues.

Table 4.1. External and internal isolations of *Scytalidium uredinicola* for three age classes of gall 3-5, 6-9, and 10+ years, sampled each month from 1994-1995.

Month	3-5 years		6-9 years		10+ years	
	Surface	**Internal**	**Surface**	**Internal**	**Surface**	**Internal**
Jan	0	0	.50	0	.70	.30
Feb	0	0	.60	0	.80	.10
Mar	0	0	.40	0	.40	.20
Apr	0	0	.70	0	.90	0
May	0	0	.80	0	1.00	.30
Jun	0	0	.60	0	.90	.20
Jul	0	0	.30	0	.80	.10
Aug	0	0	.70	0	.60	0
Sept	0	0	.70	0	.70	0
Oct	0	0	.40	0	.80	.20
Nov	0	0	.30	0	.70	.40
Dec	0	0	.20	0	.60	.20

Values indicate the proportion of *S. uredinicola* isolated from 10 galls for each age class per month sampled. *Scytalidium uredinicola* was significantly more frequent on external tissues of 10+ -year-old galls than on both internal tissues of this age class ($P = 0.002$), and the external tissues of 6- to 9-year-old galls. ($P = 0.006$)

Galls greater than 10 years old had many cracks and fissures on the surface with at least 3-4 seasons of previously infected aecia attached. Secondary phloem for this age class was further diminished to less than 2 μm in thickness.

4.3.2 *Seasonal development*

Light microscopy revealed that *S. uredinicola* overwinters as arthrospores within surface fissures of galls 10 years and older (Fig. 4.2a). Non-parasitized tissues sampled from 3- to 5-year old galls had fewer cracks and fissures with a more continuous periderm layer formed between host tissues that had borne aecia in 1993 and tissues that would produce aecia in 1994 (Fig. 4.2b). By late April to early May, 'hyphal wefts' of the rust had formed beneath areas of tissue that had previously sporulated (True 1938), obscuring the location of the mycoparasite. However, *S. uredinicola* hyphae were present within the developing sorus, beneath both the last formed periderm and the newly developed peridial layer, as early as 24 April 1994 (Fig. 4.2c). Complete separation between host tissues that had borne aecia in 1993 and tissues that would produce aecia in 1994 occurred along the periderm layer on samples taken from galls 3 to 5 years of age (Fig. 4.2d).

SEM showed a disrupted and damaged periderm in parasitized, 10-year-old galls collected in January in areas beneath where *S. uredinicola* was located in 1995 (Fig. 4.3a). The hyphae appeared to have penetrated the periderm to overwinter in the outer secondary phloem of the gall (Fig. 4.3b). These hyphae were distinguished on the basis of their thin, slender hyphal tips and small diameter (Fig. 4.3c). By comparison, non-parasitized tissues on 3- to 5-year-old galls had tightly compacted gall rust hyphae and no evidence of other fungal hyphae present in the area of the last formed periderm or secondary phloem. The periderm layer also appeared thicker and more continuous (Fig. 4.3d). Identification of the fungus as *S. uredinicola* was based on the presence of circular arthroconidia (Fig. 4.4a), that later resulted in the formation of segmented hyphae (Fig. 4.4b).

Field examination in 1993 detected *S. uredinicola* as early as 24 April (Fig. 4.5a) and 3 May in sites 1 and 2, respectively. Due to a late spring thaw in 1994, first detection

Figure. 4.2. Overwintering and early development of *S. uredinicola*.

a. *Scytalidium uredinicola* overwintering as arthroconidia (arrows) within a crack or fissure of parasitized gall tissue sampled from a 10-year-old gall in January. Bar = 10 μm.

b. Non-parasitized 3- to 5-year-old gall sampled in January. Arrow indicates continuous periderm layer (pd) that formed at the end 1993, below which will give rise to new aecia in the spring of 1994. Bar = 10 μm.

c. *Scytalidium uredinicola* hyphae (arrows) developing beneath the last formed periderm (pd) and the newly formed peridial layer (pi) as well as deep within the unruptured aecia 24 April 1994. Bar = 60 μm.

d. Non-parasitized 3- to 5-year-old gall sampled in April. Arrow indicates complete break in the periderm layer (pd) that formed at the end 1993, below which gives rise to new aecia in 1994. Bar = 10 μm.

Pd
Pd
Pd
Pd

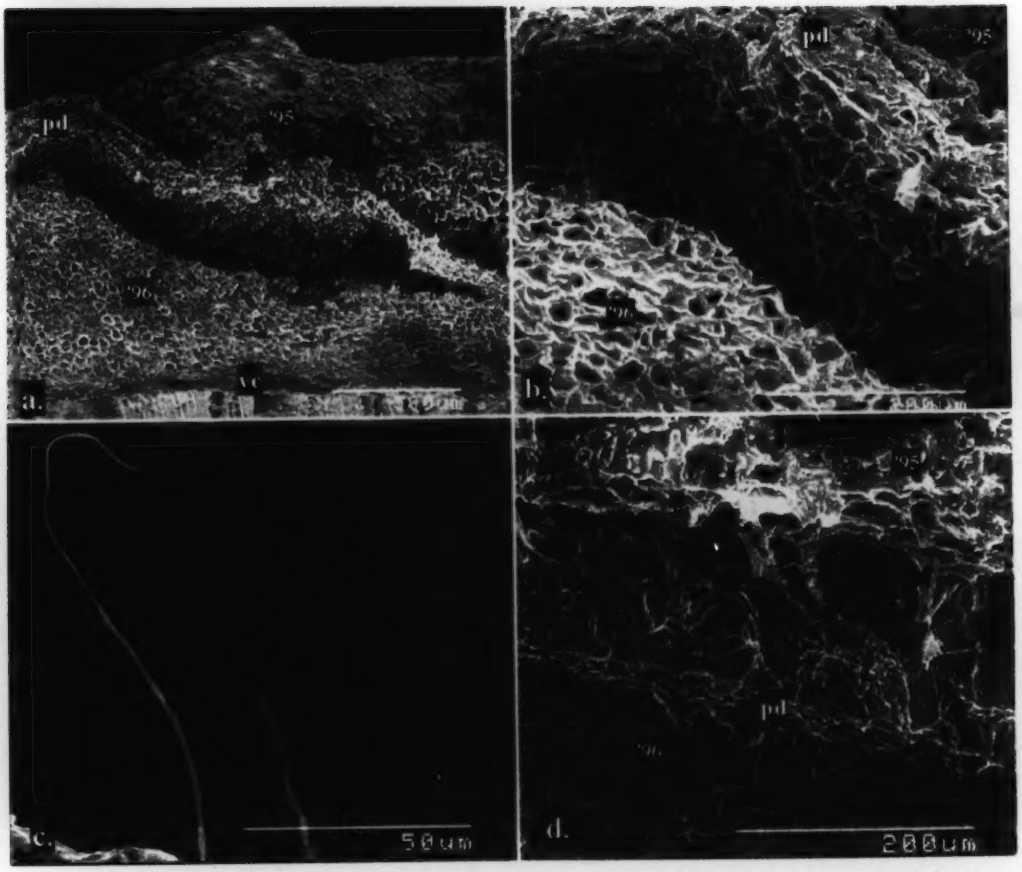
Figure. 4.3. Scanning electron micrographs of parasitized and non-parasitized gall tissues collected in January.

a. Fifteen-year-old January gall parasitized by *S. uredinicola.* Note that the 1995 ('95) aecium has not been exfoliated. The periderm (pd) appears discontinuous. A new aecium will develop from the rust infected secondary phloem above the vascular cambium (vc) in tissues produced in 1996 ('96). Bar = 500 μm.

b. A magnified view of the area beneath the disrupted periderm (pd). *Scytalidium uredinicola* hyphae are located in the area beneath the tissue that previously had borne aecia ('95) and are emerging from the tissues that will bear new aecia in the spring ('96). Bar = 200 μm.

c. Slender hyphae of *S. uredinicola* overwintering between tissue that previously supported aecia 1995 ('95) and the last formed periderm. Bar = 50 μm.

d. Non-parasitized gall tissues (3-5 years old) collected in January showing a continuous thick layer of periderm (pd) and the complete separation between the tissues that supported aecia in 1995 ('95) and those tissues that will support aecia in 1996 ('96). Bar = 200 μm


pd
95
96
vc
a.
pd
95
96
b.
c.
50um
95
pd
96
d.
200um

occurred on 13 May and 20 May in the two sites. The first detection of *E. obliquus* followed the initial detection of *S. uredinicola* in both years. Adult beetles were detected on galls on 4 May and 13 May 1993, and again on 15 May and 3 June 1994 (Fig. 4.5b). *Cladosporium gallicola* appeared considerably later than *S. uredinicola.* It was first observed on galls on 10 June and 17 June, 1993 and on 21 June and 30 June, 1994 in site 1 and site 2, respectively. On all ten galls, *S. uredinicola* reappeared in 1994 at the same location on the gall where it had been positively identified in 1993. Further, samples taken after three sporulating seasons had active *S. uredinicola* in the same areas identified in 1993 (Fig. 4.5c).

4.3.3 *Rust spore viability*

Viability of rust spores collected from parasitized areas of the galls, averaged over stands, years, and collection dates, was 5.5%. By contrast, viability from non-parasitized areas of the galls averaged 90% (Fig. 4.6).

4.4 DISCUSSION

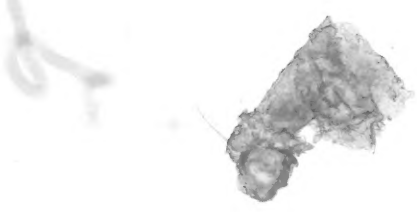
Since *S. uredinicola* disarticulates as arthrospores upon maturation, it is unlikely that these spores are wind disseminated (Cole and Kendrick 1969; Kuhlman *et al.* 1976). Hence, *E. obliquus* likely is required for the initial introduction of *S. uredinicola* onto galls previously free of hyperparasites (Currie and Hiratsuka 1996). However, even if beetles target galls of all ages, it is apparent that not all galls become parasitized. There are several possible reasons why 3- to 5-year-old galls had no *S. uredinicola.* First, periderm formation appeared abundant and continuous over much of these younger galls, resulting in the complete exfoliation of aecia prior to rust sporulation the following spring. Further, aeciospore production in this age class was so prolific that it may have limited *S. uredinicola* colonization. Even if the sorus had become parasitized, the mycoparasite might either have been expelled or exfoliated from the gall since rusts are typically most aggressive on young hosts (Peterson and Jewell 1968).

As the gall ages, more openings in the periderm due to expansion and insect feeding may serve as infection courts for the arthrospores of *S. uredinicola.* The colonized aecia

Figure. 4.4. Light micrographs of *S. uredinicola*.

a. Positive identification of *S. uredinicola* was based on the circular arthroconidia and segmented hyphae (s). Note the parasitized *E. harknessii* aeciospore (w). Bar = 50 μm.

b. Segmented hyphae and arthrospores of *S. uredinicola* (s) and an aeciospore of *E. harknessii* (w). Bar = 50 μm.



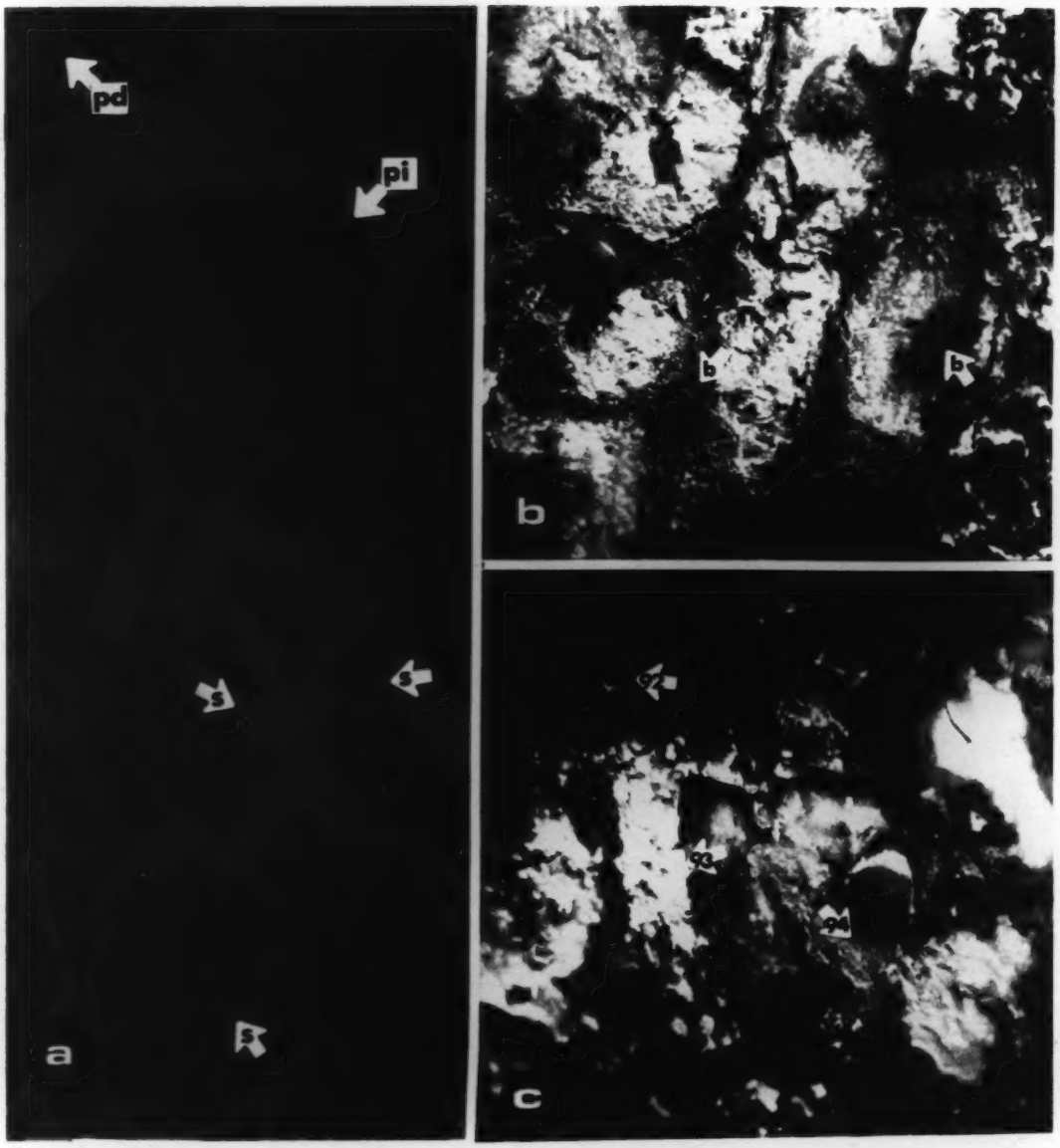
a
b

Figure. 4.5. Initial detection and seasonal recurrence of *S. uredinicola* and beetle emergence.

a. First detection of *S. uredinicola* on 24 April 1994. The periderm (pd) was continuous and the peridial layer (pi) had not yet ruptured. *Scytalidium uredinicola* was apparent deep within the intact sorus. Bar = 50 μm.

b. *Epuraea obliquus* (b) first observed feeding on sporulating aecia of *E. harknessii* on 7 May 1994. The insect vector was first observed after first detection of *S. uredinicola.* Bar = 10 mm.

c. *Scytalidium uredinicola* in the same area on the gall in three successive sporulating seasons from '92-'94. Bar = 10 mm.


pd
pi
s
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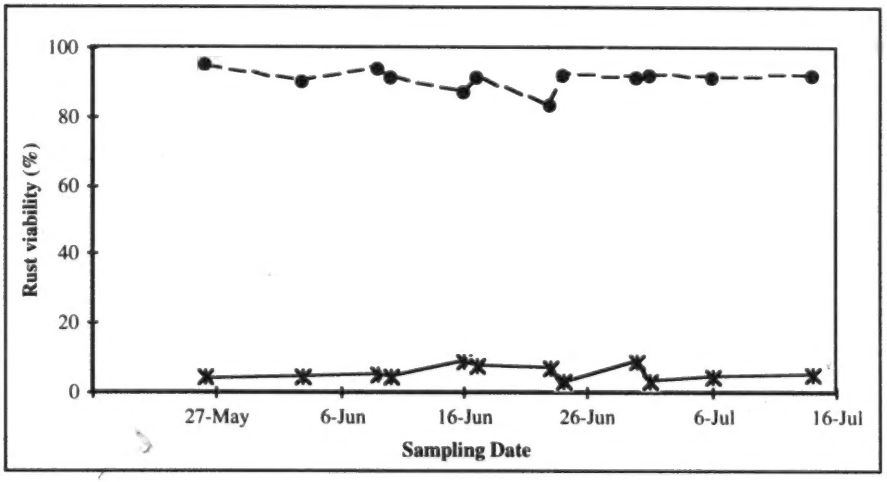
a
b
b
b
c

Figure 4.6. 1993-1994 combined field results of *Scytalidium uredinicola* and its effect on gall rust viability.



Viability of gall rust spores from non-parasitized galls (●) and parasitized galls(✱). *S. uredinicola* reduced gall rust viability on average by 85%.

that remain attached to 6- to 9-year-old galls may therefore be important sources of inoculum due to their close proximity to the newly emerging rust sori in the following spring. Moreover, emerging *E. obliquus* are presumably attracted to these sources, thereby facilitating the introduction to and subsequent movement of arthrospores over the gall surface and throughout the stand (Currie 1995; Currie and Hiratsuka 1996). Since *S. uredinicola* was not recovered from the internal tissues of 6- to 9-year-old galls, residual inoculum on the external surface of the gall is likely responsible for the year-to-year persistence of the fungus in this age class. Galls greater than 10 years old had more colonized aecia remaining than younger galls. In addition, hyphae of *S. uredinicola* had penetrated deep beneath the periderm prior to dormancy. These hyphae likely overwintered in the secondary phloem and began parasitizing the rust as soon as aecia began to develop in the spring. Cold dormant temperatures do not appear to affect arthrospore viability of *S. uredinicola* from season to season (Chakravarty and Hiratsuka 1995; Moltzan *et al.* 1995). Results of this study indicate that *S. uredinicola* is viable in each of the 12-months sampled on galls 6 years and older (Table 4.1).

Scytalidium uredinicola did not kill the vascular cambium of the gall as did other fungi such as *Gibberella lateritium* (Nees.) Synder and Hansen, *Nectria fuckeliana* Booth, and *Diplodia pinea* (Desm.) Kicks (Byler *et al.* 1972*a*) and therefore may not inactivate galls. This is in contrast to previous reports that suggest complete inactivation of galls may be directly attributable to *S. uredinicola* (Currie and Hiratsuka 1996; Tsuneda *et al.* 1980). While aeciospore inactivation is significant from year to year, *S. uredinicola* may only play a minor role in the actual death of the gall over time.

Microtome sections of parasitized galls taken 24 April 1994 (Fig. 4.5a) showed that *S. uredinicola* was active within an intact sorus prior to and at the onset of gall rust sporulation. This activity also occurred before significant beetle emergence, indicating that once established the fungus can persist on the gall and infect emerging sori without the aid of the insect vector. Further, by becoming active earlier in the spring than *C. gallicola, S.*

uredinicola may have gained a competitive advantage in resource capture. We have shown that rust spore production reaches a maximum during the first two weeks of June in west-central Alberta (Chapter 2). Results of this investigation indicate that *S. uredinicola* reduced viability of aeciospores by 85% over two seasons of sporulation (Fig. 4.6). Therefore, this mycoparasite is a good choice for a biological control agent, since it reduces germination during the period of highest host susceptibility (Chapter 2).

Byler and co-workers (1972*a*) reported that many gall-invading fungi need an appreciable period of time before populations can successfully build-up in a stand. They concluded that environmental factors unique to the stand such as temperature, moisture, and relative humidity determine which species of mycoparasite increase on the gall. It is our contention that in addition to these factors, changes that occur during gall development also contribute to the presence or absence of mycoparasites. Results of this study confirm that *S. uredinicola* is self-sustaining and perennial on galls greater than 10 years old (Table 4.1) within colonized aecia, tissues of the last formed periderm, and in the living secondary phloem (Fig 4.2, Fig. 4.3) By contrast, although *S. uredinicola* was frequently present on or within exfoliating aecia of 6- to 9-year-old galls, it was not recovered from tissues beneath the periderm. The fact that *S. uredinicola* did not establish on 3- to 5-years-old galls supports our previous report (Chapter 3), and suggests that a delay of 6 years is needed before the mycoparasite can become perennially established. This delay may be a significant barrier to the successful establishment of *S. uredinicola* in a stand.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Spore availability may be one of the most important factors influencing the period of infection of pines by western gall rust. Spore production which initially lagged behind host susceptibility may be important in limiting infection at the beginning of the growing season. At the end of the growing season, spore production decreased prior to, or simultaneously with, the decrease in host susceptibility. Jointly, the decline in spore production and decrease in host susceptibility would greatly reduce the amount of infection once shoots reach about 90% of final elongation. Shoot morphogenesis in greenhouse-grown seedlings paralleled that observed in the field, justifying the use of greenhouse data to predict susceptibility in the field. The pattern of periderm formation from the base of the shoot upward may help to explain why gall location and infection frequency change as the shoot elongates, with infection first increasing as shoots increase in target size and then declining as periderm develops.

The importance of periderm as a protective barrier against the environment, insects, and fungal pathogens has been well documented in conifers (Lewinsohn *et al.* 1991; Mullick and Jensen 1973; Struckmeyer and Riker 1951; True 1938). There is evidence to suggest that periderm plays a significant role in resistance to pine stem rusts (Miller *et al.* 1976; True 1938; Walkinshsaw 1978). The fact that susceptibility was high prior to periderm formation supports the hypothesis that this tissue is important in limiting infection (True 1938). The findings of Chapter 2 are consistent with the report that susceptibility differed between two stages of shoot development (Kojwang and van der Kamp 1992), but provide greater detail on the relationship between infection and shoot elongation.

Chemical control of western gall rust may be more effective if treatments are applied after spore release and prior to shoots reaching 90% of their final length. *Scytalidium uredinicola* has some potential for use in biological control of gall rust given that sori without evidence of the mycoparasite had 90% rust germinability. Conversely, on galls where *S. uredinicola* parasitism occurred, rust spore germination was reduced to 5.5%.

Scytalidium uredinicola was also active prior to spore release, and thus would be capable of significantly reducing infection from the very beginning of the growing season.

It is apparent that older galls in west-central Alberta are seldom free from invasion by other fungi (Currie and Hiratsuka 1996; Hiratsuka *et al.* 1987; Tsuneda and Hiratsuka 1979; Tsuneda and Hiratsuka 1981; Tsuneda *et al.* 1980). However, the extremely high levels of *S. uredinicola* previously reported (Currie and Hiratsuka 1996; Tsuneda *et al.* 1980) may not be occurring uniformly throughout this region. The findings of Chapter 3 indicate that the frequency of *S. uredinicola* was associated with gall age and size, in spite of other important factors such as microclimate, occurrence of the insect vector, fungal interactions, etc. that would influence the likelihood of hyperparasitism. Although gall age and size were positively correlated, the odds of hyperparasitism increased with gall age and infection, with the odds of recovering *S. uredinicola* increasing by a factor of 1.3 for each year of growth, even for galls of similar size.

Isolations from internal and external gall tissue confirmed that *S. uredinicola* is self-sustaining and perennial on galls greater than 10 years old. The hyperparasite was found within colonized aecia, tissues of the last formed periderm, and deep within the living secondary phloem. By contrast, galls 6 to 9 years old were frequently colonized by *S. uredinicola* on or within exfoliating aecia and never recovered from tissues that lie beneath the periderm. The failure of *S. uredinicola* to become deeply established on 3- to 5-year-old galls suggests that a delay of 6 years is needed before this mycoparasite can become perennially established within the gall rust pathosystem.

There are a number of reasons why parasitism might increase with gall age. Developmental changes associated with gall aging may increase infection frequency. As aecia become active in the spring they push up as more aeciospores form below, causing the host tissues bearing aecia from the previous year to exfoliate as sporulation begins. As sporulation ends, the formation of a new periderm is completed by fall and separation of aecia-bearing tissues begins, culminating before winter (True 1938) or prior to March (Chapter 4). It is

possible that complete exfoliation of the aecia-bearing tissues on young (2- to 5-year-old) galls prevents *S. uredinicola* from building up on the surface of the gall. Conversely, mature (> 6-year-old) galls form many sequent periderms and retain most of their exfoliating tissues, allowing the mycoparasite to accumulate. *Scytalidium uredinicola* has not been observed in heavily infected young stands even on large-diameter galls (personal observation), and results from Chapter 3 indicate that the hyperparasite does not become established early in gall rust epidemics. This lag time in establishment may be an important obstacle to the use of *S. uredinicola* as a biological control agent.

In addition to gall age, the odds of recovering *S. uredinicola* also increased (by a factor of 2.1 for each 1 cm increase) with gall size, even for galls of similar age. *Epuraea obliquus* is instrumental in the initial introduction to, and subsequent movement of arthrospores over the gall surface, and throughout the stand (Currie 1995; Currie and Hiratsuka 1996). As galls increase in size, for any given age, main stem galls become larger than branch galls and hence some of the apparent effect of gall size may be a consequence of differences in where the galls formed.

The weak association between gall rust severity within stands and the incidence of *S. uredinicola* implies that the occurrence of the hyperparasite does not necessarily increase with increasing levels of western gall rust. However, it is still possible that the hyperparasite is acting as a density dependent constraint on the rust population. Further efforts to resolve this issue are required, since density dependent and density independent pathosystems may not respond as expected to different disturbances (Reeve 1988).

Western gall rust will continue to pose a threat to North American hard pines. The potential for spread to other areas of the world should intensify efforts to minimize this disease. While there are known control measures, none to date have proven totally effective. By further defining when infection occurs during the growing season, a specific time frame for successful application of chemical or natural control agents was established. Biological control strategies using aggressive mycoparasites are appealing since the control is

target specific and occurs naturally within the pathosystem. However, in the case of *S. uredinicola*, early establishment in the life history of a rust epidemic will be needed to ensure long term control.

More research is required to establish the link between *S. uredinicola* and western gall rust to corroborate the arguments for its use in biological control. Critical investigation on the factors that influence its early establishment will be useful in the effort to promote this naturally occurring control in the pathosystem. In addition, the horizontal and vertical spread of *S. uredinicola* should be quantified as a discrete measure to assess its specific contribution to control, independent of other naturally occurring parasites of western gall rust. Finally, fungal interactions may account for when and where *S. uredinicola* will become established, hence further studies to resolve the connection between gall-associated fungi are required to better understand the diversity of fungal communities as they occur on the gall.

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Appendix 1. Second order polynomials fitted through shoot elongation 1992-1993.